STUDIES ON THE IMPROVEMENT OF LYSINE PRODUCTION IN THE GENERA BREVIBACTERIUM AND CORYNEBACTERIUM

BY

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ERRATA

In spite of the care taken, certain typing and other errors have unfortunately occurred in the text. To avoid possible misinterpretation, the following errata should be noted.

Page 7, line 5: "advantages" should read "advances".

Page 16, line 12: "α-ketroglutaric acid" should read "α-ketoglutaric acid".

Page 18, line 11: "theonine" should read "threonine".

Page 26, line 8: "thioesther" should read "thioether".

Page 55, line 7: "sights" should read "sites".

Page 60, line 8: "obtained" should read "contained".

Page 77: In the table, enzyme activities are expressed in international units. One unit, U, is the enzyme activity which transforms 1 μmole substrate per minute under the experimental conditions.

Page 109: Title of Appendix B was omitted. It should read "Media".

Page 111, line 19: "N_2" should read "N".

Page 116: Title of Appendix C was omitted. It should read "Amino acid analysis of various amino acid sources".

Page 128: Reference to Lurie, J. (1980) should read "South African Food Review 9 : S136-139."

CONTENTS

ABSTRACT

A programme was undertaken to obtain high lysine producing bacteria by mutation of selected wild type strains. Overproduction of glutamic acid under suitable physiological conditions, viz biotin limitation, was chosen as a good indication of the potential of wild type bacteria for improvement in lysine production by mutation.

Brevibacterium lactofermentum ATCC 13869 was found to produce the highest amount of glutamic acid under the conditions used. Homoserine and leucine auxotrophic mutants were obtained from this organism and tested for ability to produce lysine. The combination of homoserine and leucine auxotrophy was found to be most effective in overcoming several of the control mechanisms present in the lysine biosynthetic pathway. Lysine production was increased approximately forty fold over the wild type B.lactofermentum.

Lysine analogue resistant strains were obtained by further mutation, and lysine production was increased by 20%.

The activity and properties of aspartate kinase, a key enzyme in biosynthesis and control of lysine production, was determined to elucidate the nature of the analogue resistance. Although resistance to feed-back control by lysine and threonine was not responsible for the improvement in lysine production, a considerably higher enzyme activity was found. As a result of the enzyme study a possible novel regulatory system in the lysine biosynthetic pathway of B.lactofermentum ATCC 13869, and the mutants derived from it, was indicated.

Environmental optimization studies were undertaken on potentially suitable mutants in order to increase lysine production still further. Fermentation media were improved and a series of fermentations were conducted under precisely controlled conditions in 12 and 20 litre laboratory scale fermenters. The most successful attempt incorporated incremetal feeding of yeast extract and glucose to an S-(2-aminoethyl)-L-cysteine resistant double auxotrophic mutant. A yield of 32 mg/mℓ L-lysine.HCl was obtained after 73 hours.

ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor Dr. T.G. Watson for his unfailing advice, constant encouragement and enthusiasm expressed throughout the duration of this study.

I would also like to express my gratitude to Professor D.R. Woods for his advice concerning the preparation of this manuscript, and to Ilze Meyer for her technical assistance.

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE FERMENTATION INDUSTRY

The fermentation industry is based on the chemical transformation of organic compounds by microorganisms into economically important products. Some fermentation processes are very old. The ability of yeast to make alcohol in the form of beer was known to the Sumerians and the Babylonians before 6 000 B.C. and the cultivation of acetic acid bacteria to make vinegar, as well as lactic acid bacteria to preserve milk is rooted deep in antiquity. (Demain and Soloman, 1981).

Fermentation as a source of industrial chemicals rather than food or beverage products is, however much more recent and really only started in 1881 with the construction of the first factory for manufacturing lactic acid (Perlman, 1969). Until the acetone-butanol process was commercialized during the first world war this was the only fermentation process operated on a large scale.

Today alcoholic beverages, solvents, organic acids - including amino acids, enzymes, nucleic acids, transformed steroids, biomass as well as approximately a hundred antibiotics are all made industrially by fermentation. In the United States, the annual value of the products from fermentation is estimated in tens of billions of dollars and in Japan the fermentation industry provides approximately 3% of the G.N.P. and 10% of the nations total tax income (Arima, 1977). In South Africa where the industry is much smaller and is centered largely around potable alcohol production the volume of the products still exceeds R600 million annually. (Lurie, 1980).

Worldwide the general trend has appeared to be one of rather continuous expansion. There has, however, been a shift in emphasis leading to the virtual extinction of traditional fermentations producing organic solvents such as ethanol, acetone and butanol, in the wake of the cheap petro-chemical era of the fifties and sixties.

Attention became directed more to products and processes which yielded complex organic molecules of high value, eg. antibiotics and enzymes or products which could not be made easily by chemical synthesis eg. citric acid, monosodium glutamate and lysine (Perlman, 1969). Undoubtedly recent advantages in genetic manipulation especially using recombinant DNA techniques, protoplast fusion and gene amplification will allow microorganisms to produce products which they were previously unable to do, even when conventional mutational techniques were applied. However, a revival in the more traditional fermentations may also occur as a result of current interest in the use of biorenewable resources to replace the ultimately exhaustable and increasingly expensive petrochemicals.

1.2 INDUSTRIAL PRODUCTION OF AMINO ACIDS BY FERMENTATION

1.2.1 General

Interest in amino acid production began at the turn of the century. Konbu which is a kelp like seaweed was widely used as an important traditional seasoning source in Japan. In 1908 the taste of konbu was identified as being due to L-glutamic acid by Ikeda. (Hirose and Okada, 1979). Based on this discovery, the industrial production of monosodium L-glutamate was initiated by the Ajinomoto Co. in 1909. At that time L-glutamic acid was produced by acid hydrolysis of wheat gluten or soybean protein. A half century after this discovery it was reported that considerable quantities of L-glutamic acid accumulated in bacterial cultures (Kinoshita et al, 1957).

The research and development carried out mainly in Japan resulted in the successful and economical production of L-glutamic acid by the fermentative process. The significance of the establishment of microbial production of L-glutamic acid cannot be over-estimated. Such essential metabolites as amino acids or nucleotides were considered not to be accumulated in microbial cultures due to regulatory mechanisms in the cell. The discovery of L-glutamic acid fermentation stimulated a wide

variety of research aimed at the isolation of wild strains and the genetic derivation of mutants which could accumulate large amounts of primary metabolites.

Fortunately fundamental knowledge about biosynthetic pathways of amino acids and their regulatory mechanisms had already been elucidated to some extent.

The production of monosodium glutamate (MSG) far exceeds that of any other amino acid and is currently around 300 000 tons per year (Everleigh, 1981) with Japan still ranking as the number one producer. MSG is, however, not the only amino acid to be produced by fermentation on a large scale. Some 40 000 tons of L-lysine are produced annually, mainly as an animal feed supplement. Methionine is also an important feed supplement although it is synthesized chemically.

Other amino acids used commercially although in relatively small quantities (table 1) include L-arginine, L-histidine, L-isoleucine, L-leucine, L-phenylalanine, L-proline, L-serine and L-threonine. These are all components of intravenous nutrient solutions. In addition L-arginine, L-cysteine, L-glutamine, L-histidine and L-alanine are used in the pharmaceutical field and the food industry.

Amino acid derivatives are also used in the chemical industry. They are applied in cosmetics, synthetic leathers, surface active agents, fungicides and pesticides (Hirose and Okada, 1979). Thus in order to meet these diversified demands almost all of the amino acids are now commercially produced although not all by fermentation (table 1). The production methods developed to date are:

(i) Protein hydrolysis methods.

In the case of lysine production a good deal of work was done on the cultivation of microorganisms containing lysine-rich protein from which L-lysine could be obtained by hydrolysis. Many of these microorganisms also accumulated some lysine in the culture medium.

Amino acids	Industrial production methods*	Annual output (tons/year)	Main uses
L-Ala	C-3	10 - 50	Flavouring
DL-Ala	B, C - 1	150 - 200	Flavouring
L-Arg	C - 1, A	200 - 300	Infusion, therapeutic
L-Asp	C - 3	500 - 1000	Therapeutic, flavouring
L-Asn	B	10 - 50	Therapeutic
L-CysH	A	100 - 200	Improver of bread quality, antioxidant
L-Glu	C - 1	200 000	Seasoning
L-Gln	C - 1	300	Therapeutic
Gly	B	5000 - 6000	Sweetner
L-His	C - 1	100 - 200	Therapeutic
L-Ile	C - 1	10 - 50	Infusion
L-Leu	A	50 - 100	Infusion
L-Lys	C - 1	15000 - 20000	Feed additive
DL-Met	B	70000	Feed additive
L-Phe	B	50 - 100	Infusion
L-Pro	C - 1	10 - 50	Infusion
L-Ser	C - 2	10 - 50	Cosmetic
L-Thr	C - 1, B	50 - 100	Food additive
L-Trp	B	50 - 100	Infusion
L-Tyr	A	50 - 100	Infusion
L-Val	C - 1	50 - 100	Infusion

Table 1 (Hirose and Okada, 1979)

* Key to methods A : protein hydrolysis method

B : chemical synthesis

C : methods employing microbial activities

C-1 Direct production from glucose or other carbon-sources

C-2 Precusor addition methods

C-3 Enzymatic methods

Production methods, annual outputs and most important uses of amino acids

(ii) Chemical synthesis methods.

DL-methionine, DL-alanine and glycine still depend on chemical synthesis methods. It was found that when optical resolution was not necessary then the chemical synthetic method remained more economical. For example although natural methionine in proteins is present in the optically active L-isomer it can be injested in the D-form and converted in vivo to its biologically active L-form. Therefore complex and costly separation of the D and L isomers produced by chemical synthesis is not required.

(iii) Microbiological methods (fermentation).

These are divided into three groups:

(a) Direct production of amino acids from a carbon source such as glucose, where the organism synthesizes amino acids and accumulates them in the medium as in the case of L-glutamic acid, L-lysine, L-isoleucine, L-proline and L-valine among others.

(b) Precursor addition methods where the organism synthesizes amino acids from immediate metabolites such as L-serine accumulation from glycine.

(c) Enzymatic methods. This involves the enzymatic production of amino acids from their immediate substrates and is exemplified in L-aspartic acid synthesis from fumaric acid and ammonia by the aspartase of Escherichia coli (Hirose and Okada, 1979).

At present most commercial amino acids result from the microbial method. Only L-cysteine and L-hydroxyproline are now extracted from natural protein hydrolyzate and when optical resolution is not necessary the chemical synthetic method is employed.

1.2.2 Lysine

Of the twenty or so amino acids contained in animal tissue, ten are commonly recognized as being "essential" for monogastric animals such as humans, poultry and swine. That is, these amino acids must be included in their diets. Of the ten amino acids, only methionine and lysine have commercial markets that are substantial enough to justify their synthesis and manufacture in volume by the chemical industry and well over 90% of both products are used to supplement animal feed rations.

Interest is also being shown, however, in the application of lysine as a human dietry supplement particularly in the underdeveloped overpopulated regions of the world where the chief dietry supplements are deficient in these amino acids. (Daoust, 1976; Kahn and Vora, 1977).

DL-Methionine is produced by chemical synthesis and L-lysine by fermentation (Section 1.2.1). In the production of L-lysine many sources of carbon may be used and the choice is determined by availability and relative cost. The most common source is cane molasses. Current industrial production is a one-step batch process using mutants of suitable coryneform bacteria and related genera (section 1.2.3). In this process a solution of aqueous molasses (or other suitable carbon source), hydrolyzed soybean meal (or other inexpensive complex nitrogen source such as corn steep liquor), ammonium compounds and inorganic salts are fermented under aerobic sterile conditions.

Major free-world producers of L-lysine are Japan, South Korea, France and Mexico. L-Lysine is not at present manufactured in South Africa. Although local usage is currently very low, a significant increase could be envisaged should fishmeal, a lysine rich component of many animal feedstuffs become less available or prohibitively expensive in the future. In planning for this eventuality the present study was initiated.

1.2.3 "Coryneform group of bacteria"

These glutamic acid producing bacteria in particular are extremely important industrially in the fermentative production of glutamic acid and certain amino acids especially lysine. Some coryneforms are also used in the microbial conversion of steroids and in the ripening of certain cheeses. (Keddie, 1978).

The "coryneform group of bacteria" includes Corynebacterium, Arthrobacter (with the related genera Brevibacterium and Microbacterium as genera incertae sedis) Cellulomonas and "tentatively" Kurthia. They are Gram-positive rods which in the case of Corynebacterium may be slightly curved while for Brevibacterium they are typically short unbranched rods.

Yamada and Kamagata in 1970 (Rogosa et al, 1974) found that the G + C moles % in named strains of Brevibacterium varied from 46,6 to 70,5. However, glutamic acid producing bacteria whether they were previously named Brevibacterium, Corynebacterium or Micrococcus species all had a narrow range of G + C values with a mean value of 53,1 $\pm$ 1,3. All had a 'snapping mode' of cell division and had DAP (α,ε diaminopimelic acid) in the cell walls. Among these were the following strains: B.flavum (ATCC 14067) B.lactofermentum (ATCC 13869) and Micrococcus glutamicus (ATCC 13032) (Syn. Corynebacterium glutamicum.)

They are all natural biotin auxotrophs, which is one of the most important factors contributing towards their ability to produce glutamic acid. (section 1.3.1). (Rogosa et al, 1974).

1.3 MICROBIAL STRAINS EMPLOYED IN AMINO ACID PRODUCTION

A great variety of microorganisms have been isolated or suitably mutated for amino acid production. They may be classified into three groups (Hirose and Okada, 1979): (i) wild type strains, (ii) auxotrophic mutants (iii) regulatory mutants. Examples from each of

these three categories will be discussed in detail with a view specifically towards lysine production.

1.3.1 Wild type strains

The economical production of L-glutamic acid is of considerable commercial importance since the monosodium salt is highly useful as a flavour-enhancing agent in food preparation (section 1.2.1). According to Kinoshita et al (1957) it has been generally recognized that glutamate is one of the primary products of nitrogen metabolism in the living cell and the glutamic dehydrogenase system represents an important link between the metabolism of amino acids and carbohydrates. It is also known that the glutamate so formed is apt to be transformed rapidly into various other amino acids and proteinaceous materials. It would therefore be of importance to determine the glutamic acid production potential of organisms intended as lysine producers.

Kinoshita et al (1957) looked at the production of L-glutamic acid by various microorganisms. After screening different strains of bacteria, yeasts and fungi, they came to the conclusion that the highest level of glutamate production was obtained by a bacterium, a species of Micrococcus. Upon subsequent detailed studies it was found to be related to so called "coryneform bacteria" (section 1.2.3).

According to Kinoshita and Tanaka (1972) and Hirose et al (1978) all industrially important glutamic acid producers belong to the genera Brevibacterium, Corynebacterium and to a lesser degree to Arthrobacter and Microbacterium.

Otsuka et al (1965) compared the pathways through which glutamate is produced from glucose. They utilized Brevibacterium flavum and Micrococcus glutamicus (syn. Corynebacterium glutamicum) which are both natural biotin auxotrophs. They found no significant difference between the two bacteria, but they did find that both B.flavum and M.glutamicus produced large amounts of glutamate from glucose in biotin poor media. The amount of biotin present proved critical to glutamate production.

Tanaka et al in 1960 (Hirose et al 1978) found that the greatest amount of glutamic acid was accumulated in the culture medium when the biotin concentration was suboptimal for maximum growth. More biotin supported abundant cell growth but seriously decreased the L-glutamic acid accumulation.

Shiio et al (1963) found that when cells were grown with excess biotin they were not able to accumulate L-glutamic acid. This was due to the lack of permeability rather than a lack of biosynthesizing activity. This finding was endorsed by Otsuka et al (1965b) who discovered that when B.flavum or M.glutamicus were cultured in biotin rich medium, L-glutamate was not formed from glucose either by resting or growing cells. They also found that enzyme activities which seemed most closely related to glutamate formation did not change significantly with biotin concentration in the culture medium, while marked differences of cellular permeability to glutamate between biotin-rich and biotin-poor cells of both strains were shown.

It was demonstrated by Takinami et al (1966, 1967) that in cells whose biotin content was greater than 0,5 µg/mg of cell dry mass there were sufficient amounts of oleic acid, which resulted in a high content of phospholipid in the cell membrane. These cells excreted endogenous L-glutamic acid poorly. However cells with a biotin content of less than 0,5 µg/mg of cells, synthesized insufficient amounts of phospholipid. These phospholipid poor cells excreted endogenous L-glutamic acid.

Izumi et al (1973) and Kamiryo et al (1976) showed that acetyl-CoA carboxylase, which is a biotin requiring enzyme is involved in the biosynthesis of oleic acid and other fatty acids and that C16-C18 saturated fatty acids inhibited oleic acid synthesis by repressing acetyl-CoA carboxylase (fig. 1).

The discovery of penicillin and saturated fatty acids as antibiotin agents allowed industrial utilization of such biotin-rich raw materials as cane and beet molasses for L-glutamic acid production. It is physiologically and industrially important that these chemical agents be

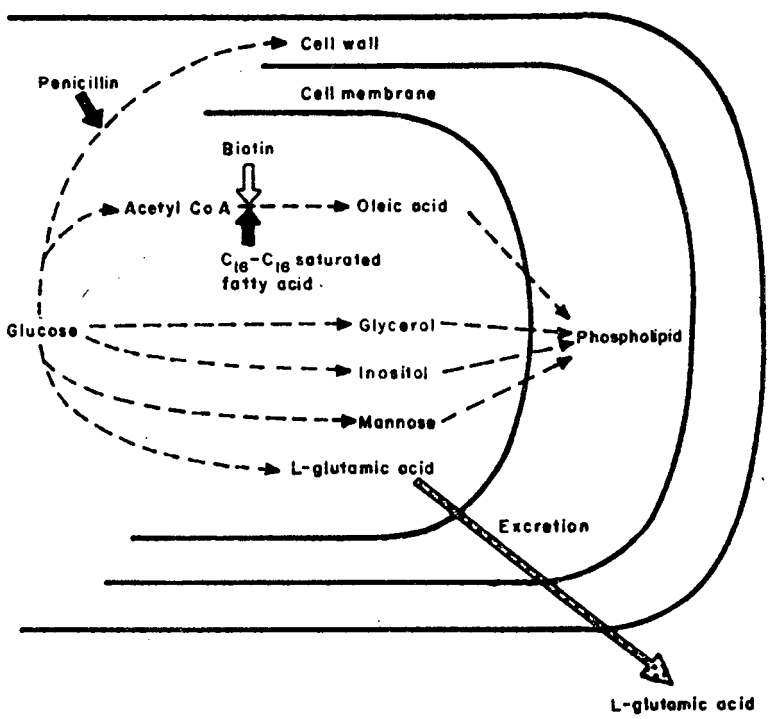


FIG. 1. L-Glutamic acid permeability in relation to phospholipid biosynthesis. Filled solid arrows show inhibition; the clear arrow shows promotion.

added to the medium during a specific phase of the cell growth. In the case of penicillin, it only promoted the excretion of L-glutamic acid in a medium of low osmotic pressure and was ineffective in a medium of high osmotic pressure, indicating that it primarily inhibited cell wall synthesis, leaving the cell membrane unprotected, thus breaking the permeability barrier causing physical damage to the cell membrane.

According to Hirose et al (1978) under optimal conditions, glutamic acid producing bacteria convert about 50% of saccharic materials into L-glutamic acid with little formation of by-products. In contrast under improper culture conditions almost no glutamic acid is produced. Instead large amounts of bacterial cells or other products such as lactic acid, succinic acid, α-ketroglutaric acid, alanine, valine, glutamine and N-acetylglutamine accumulate. The factors influencing the ratio of the products are the concentrations of biotin, ammonium ions and dissolved oxygen in the medium as well as the pH.

Wild strains were also found to accumulate various other amino acids (Hirose and Okada, 1979) such as L-valine by Paracolobacterium coliform; DL-alanine, D-alanine and L-alanine by various species of Brevibacterium, Corynebacterium, Microbacterium and Bacillus; and L-glutamine and N-acetyl-L-glutamine by species of Brevibacterium and Corynebacterium.

1.3.2 Auxotrophic mutants

In order to perform catabolic and anabolic reactions in an effective manner, regulatory mechanisms have evolved in microorganisms. In biosynthetic amino acid pathways, the main regulatory control is through feedback involving inhibition of an early biosynthetic enzyme, or repression of one or more of the biosynthetic enzymes by the final product. One of the attempts to overcome such regulatory controls was by limiting the intracellular concentration of feedback inhibitors or repressors. Usually an auxotrophic mutant was employed for this purpose. Such mutants are recognized by their growth on a complex multicomponented "complete" medium and their lack of growth on a chemically defined minimal medium. The mutants are then classified

into categories by propogation on minimal medium supplemented with amino acids (amino acid requiring), vitamins (vitamin requiring) or nucleic acid components (purine and pyrimidine requiring).

The first application of this approach to amino acid overproduction was with L-ornithine by Udaka and Kinoshita (1958). The fermentation of L-ornithine was achieved by using a citrulline auxotroph whose L-ornithine carbamyl-transferase had been genetically inactivated. When cultured with a low concentration of L-citrulline or L-arginine, the mutant accumulated much L-ornithine in the medium.

According to Casida and Baldwin (1956) one approach in producing lysine was via the conversion of its immediate precursor, diaminopimelic acid (designated DAP). This two stage process involved a lysine auxotroph of Escherichia coli which produced and accumulated DAP. The broth containing the DAP was mixed with a second culture of E.coli or with a culture of Aerobacter aerogenes, neither of which required lysine. This culture was treated with toluene, butanol, or ultrasonically to lyse the cell and release the DAP carboxylase which was needed to convert the DAP to lysine.

Kita and Huong (1958) modified this approach and used a single organism which produced the DAP and converted it to lysine, but only after the cells had been lysed. This process was on improvement but still retained the disadvantages of a two-step process.

The second and most important approach was a direct fermentation utilizing a single organism. Amongst the pioneers in this field of lysine production by selected microorganisms were Dulaney (1957) and Richards et al (1957). Dulaney found that a strain of Ustilago maydis was able to produce 400 µg/ml of extracellular lysine in shaken Erlenmeyer flasks. Richards et al (1957) screened over 600 fungi for the production of extracellular lysine on a medium containing urea, glucose and mineral salts. The highest yields of lysine of 200 to 300 µg/ml were obtained with two strains of Ustilago.

Attention was again focused on bacteria as potential lysine producers, when, while looking at the production of L-glutamic acid by various microorganisms Kinoshita et al (1957) recognized the important link between the glutamic dehydrogenase system and the metabolism of amino acids and carbohydrates. They found that the highest level of glutamate production was obtained by a species of Micrococcus. Nakayama et al (1961b) obtained nutritional mutants from glutamic acid bacteria and looked at their amino acid accumulation. At an early stage during these studies a preliminary report was published by Kinoshita et al, (1958) concerning the direct production of L-lysine from carbohydrate. This was developed utilizing a homoserine (or theonine plus methionine) auxotroph of Micrococcus glutamicus (syn. Corynebacterium glutamicum) the high glutamic acid producing microorganism. The amount of L-lysine.HCl produced during the fermentation run was approximately 14 mg/ml after 72 h and the yield based on consumed glucose as high as 29%.

This stimulated research involving the approach of producing lysine via a direct fermentation. Nakayama et al (1961) found that all homoserine-less mutants tested including those of Bacillus subtilis and E.coli showed lysine accumulation. However they found that this was minimal in comparison to that accumulated by a homoserine auxotroph of the glutamic acid producer Micrococcus glutamicus. It was also discovered that considerable lysine accumulation occured with theonine auxotrophs, isoleucine auxotrophs, leucine auxotrophs, and with isoleucine leucine double auxotrophs.

Culture Conditions

When lysine accumulation by a homoserine auxotroph of M.glutamicus was studied in detail it was found that when homoserine or threonine and biotin were in excess in the media, then lactic acid accumulated in place of lysine. In sufficient concentration of biotin and a proper amount of the required amino acids lysine accumulated, and in low biotin concentration the tendency of glutamic acid accumulation was recognized. It was also noted that homoserine and threonine specifically inhibited the lysine accumulation from glucose and ammonia by a washed cell suspension of the homoserine auxotroph of

M.glutamicus. The action of these amino acids was suggested to be by inhibition of enzyme action rather than enzyme formation.

Two L-lysine biosynthetic pathways are known in microorganisms, one in fungi and yeasts and the other in bacteria. The pathway via α-aminoadipic acid is known in the former and the pathway via α-ε diaminopimelic acid in the latter (fig. 2). This was confirmed by Nakayama and Kinoshita (1961) who demonstrated the presence of α,ε diaminopimelic acid (DAP) and DAP carboxylase in a lysine producing strain of M.glutamicus. Nakayama and Kinoshita (1961b) observed that both homoserine and threonine showed no inhibitory effect either on DAP synthesis or DAP decarboxylation; while lysine inhibited DAP synthesis specifically. This phenomenon could at that stage not be satisfactorily explained except by assuming a difference between the control mechanisms of the two mutant strains or the occurence of the pathway of lysine excluding DAP or two biosynthetic pathways of DAP.

Samejima et al (1961) while carrying out studies on fermentative production of L-homoserine using a threonine auxotroph of M.glutamicus found that L-threonine concentration affected the formation of L-homoserine and L-lysine to a great extent. Maximum productions of L-homoserine and L-lysine were attained when L-threonine concentrations were 400 µg/ml and 300 µg/ml respectively. An excess amount of threonine caused a sharp decrease in both amino acid productions. The effects of various amino acids on this fermentation were also examined. As a result two negative feedback control phenomena caused by excess threonine and methionine were observed.

According to Davis (1952) and Black and Wright (1955) both homoserine and lysine were synthesized from aspartic acid via a common intermediate denoted as "x". From these results a schematic diagram for the biosynthesis of homoserine and lysine was proposed (fig. 3).

Pardee in 1959 (Samejima et al 1961) reported that two types of negative feedback control mechanisms exist. One was "repression" which is suppression of enzyme formation and the other was "inhibition" which is suppression of enzyme activity.

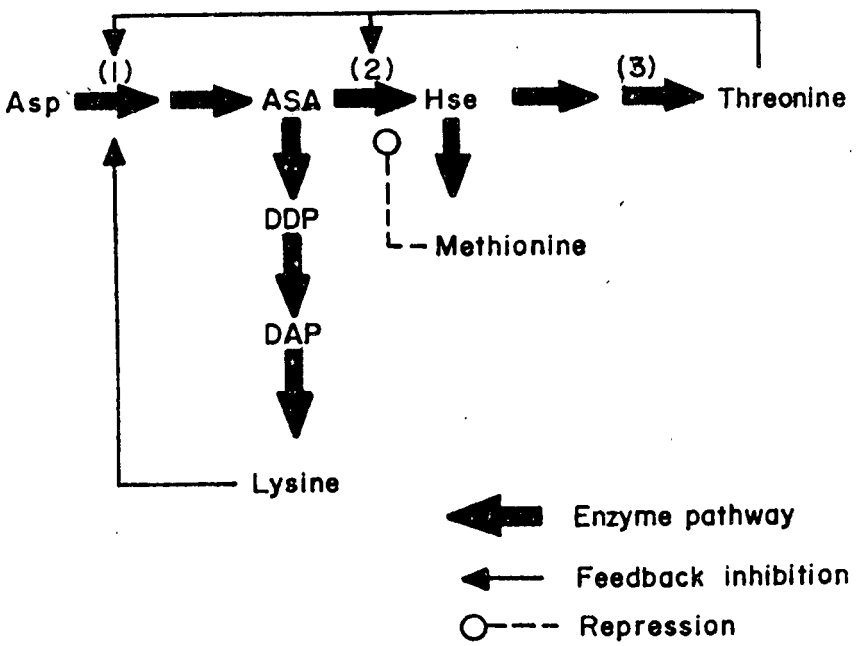


FIG. 2. ASA, aspartate semialdehyde; DDP, dihydrodipicolinate; Hse, homoserine; DAP, diaminopimelate.
(1) Aspartate Kinase, (2) homoserine dehydrogenase, (3) homoserine kinase.

Regulation in lysine biosynthesis in Brevibacterium flavum and Corynebacterium glutamicum.

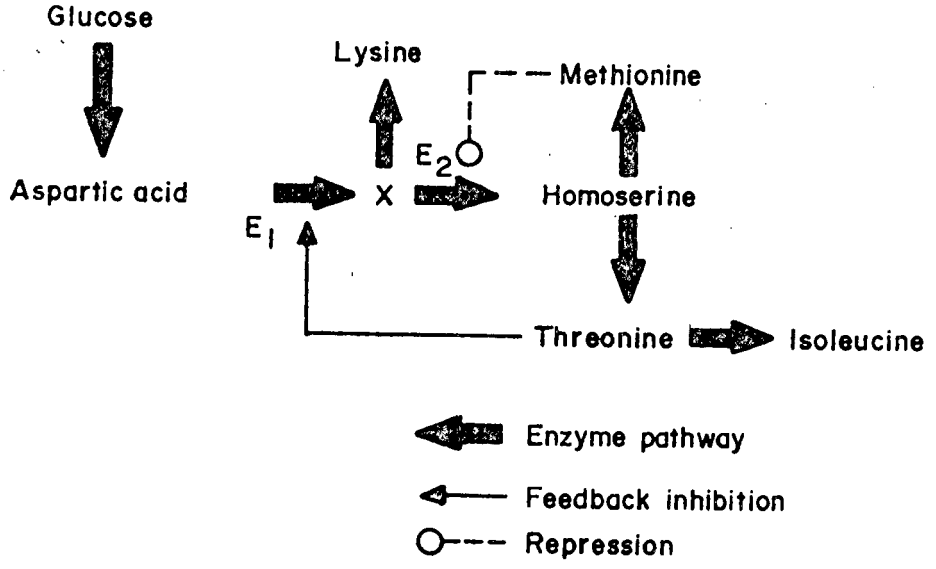


FIG. 3. Schematic diagram for the biosynthesis of homoserine and lysine, and possible sites of "negative feedback control."

Samejima et al postulated that methionine suppressed the formation of a certain enzyme system for homoserine biosynthesis designated E2. This was therefore "repression" and threonine suppressed the enzyme activities for both homoserine and lysine biosynthesis and this was "inhibition".

Samejima et al in 1961, (Nakayama and Kinoshita, 1966) investigated the effects of various amino acids on the activity of β-aspartokinase from M.glutamicus a homoserine producing, theonine requiring mutant and found that no amino acid tested had any great inhibitory effect on aspartokinase activity. It was not until 1966 when this apparent discrepency in the biochemical control mechanisms of the L-lysine pathway was explained by Nakayama et al (1966) and Nakayama and Kinoshita (1966).

They found that the β-aspartokinase activity of M.glutamicus was not inhibited by any single amino acid but was inhibited by threonine plus lysine acting in concert. Thus in the prototroph, the aspartokinase would be subject to feedback inhibition by intracellular levels of threonine and lysine. This inhibition may prevent the formation of an excessive amount of aspartyl phosphate and the amino acids such as threonine, lysine and methionine derived from it.

However in the homoserine auxotrophic mutant M.glutamicus a genetic block between aspartic semi aldehyde and homoserine dehydrogenase prevented threonine from being synthesized. Lysine was accumulated when homoserine or threonine in the medium was limited. The aspartokinase was therefore not subjected to feedback inhibition unless threonine was added as the mutant could not synthesize threonine. Lysine added externally had little effect on its accumulation.

Nakayama and Kinoshita (1966) also showed that the activity of the enzyme catalyzing the condensation of aspartic semi aldehyde with pyruvate which is the first step of the lysine branch, was not inhibited by either lysine or other amino acids. This result contrasted with that obtained with E.coli where lysine exerted a feedback inhibition on this reaction. The phenomenon that an end product, in this case lysine, exerted no feedback inhibition in the first step of the pathway for its

own synthesis proved on important advantage for the use of M.glutamicus in producing large quantities of L-lysine.

According to Shiio and Miyajima (1969) the aspartokinase which acts in the first step of L-lysine, L-threonine and L-methionine biosynthesis in B.flavum receives concerted feedback inhibition by threonine plus L-lysine. This agrees with the results obtained with M.glutamicus and reported by Nakayama et al (1966) where a similar occurrence was observed.

Stadtman et al in 1957 (Tosaka and Takinami, 1978) reported that E.coli contained at least two different and separable aspartokinases. One enzyme is specifically and competitively inhibited by lysine and the other is specifically and competitively inhibited by threonine. They also found evidence for the existance of a third aspartokinase which is specifically repressed by methionine.

However, Tosaka et al (1978b) found that in B.lactofermentum the aspartokinase was inhibited 45% by lysine or threonine at 1 mM respectively and about 80% when threonine and lysine were simultaneously added at 1 mM each. Thus the aspartokinase in B.lactofermentum was inhibited by a single or simultaneous addition of lysine and threonine. Thus this type of regulation differed from that of E.coli, B.flavum and C.glutamicum.

Although the lysine biosynthetic pathway has been proven similar in all bacteria, its regulation is found to differ from bacterium to bacterium.

The first specific enzyme in lysine biosynthesis, DDP synthetase was found not to be inhibited by lysine in B.lactofermentum. However, Tosaka et al (1978c) while investigating the effect of L-leucine on L-lysine production found that the production of L-lysine was inhibited specifically by the addition of excess L-leucine and this was accompanied by the repression of dihydrodipicolinate synthetase (DDP). L-Leucine was also found to inhibit α-isopropylmalate (IPM) synthetase which was the first specific enzyme in leucine biosynthesis, while lysine caused complete reversion from its inhibition. This is illustrated in fig.

4. L-Valine was found to release L-lysine production from the inhibition by L-leucine. It is of interest to note that DDP synthetase in B.lactofermentum was controlled by an end product of a different biosynthetic pathway.

1.3.3 Regulatory mutants

As illustrated in 1.3.2 the utilization of auxotrophs can serve as a means to overcome inhibition and/or repression. However not all amino acids for protein synthesis can be over-produced by this method because they are themselves inhibitors and/or co-repressors. In cases where auxotrophs are not effective a second technique is employed of artificial disorganization: regulatory mutants. The first evidence of this was reported as analogue resistant mutants by Adelberg (1958). While studying mutants of Escherichia coli resistant to thienylalanine, an analogue of phenylalanine, he discovered that such resistant strains were able to excrete large amounts of phenylalanine and, in some cases, smaller amounts of tyrosine.

Thus with its genetically inactivated regulatory site, the mutant may be desensitized from feedback inhibition and thus overproduce the end product. Derepressed mutants having genetically inactivated apo-repressor or operator functions can likewise produce sizeable quantities of end product.

One of the most successful examples of the application of regulatory mutants to amino acid fermentation was in the production of L-lysine by selected bacteria. Unlike the regulatory mechanism in E.coli, the regulation of L-lysine synthesis by Brevibacterium was relatively simple, as only the aspartate kinase was sensitive to feedback inhibition by lysine. (fig. 2).

Regulatory mutants were obtained by the isolation of L-lysine analogue resistant mutants whose growth was not inhibited by the lysine analogue. The analogue acts as a feedback inhibitor on aspartate kinase and thus does not allow growth of the parent strain. Regulatory

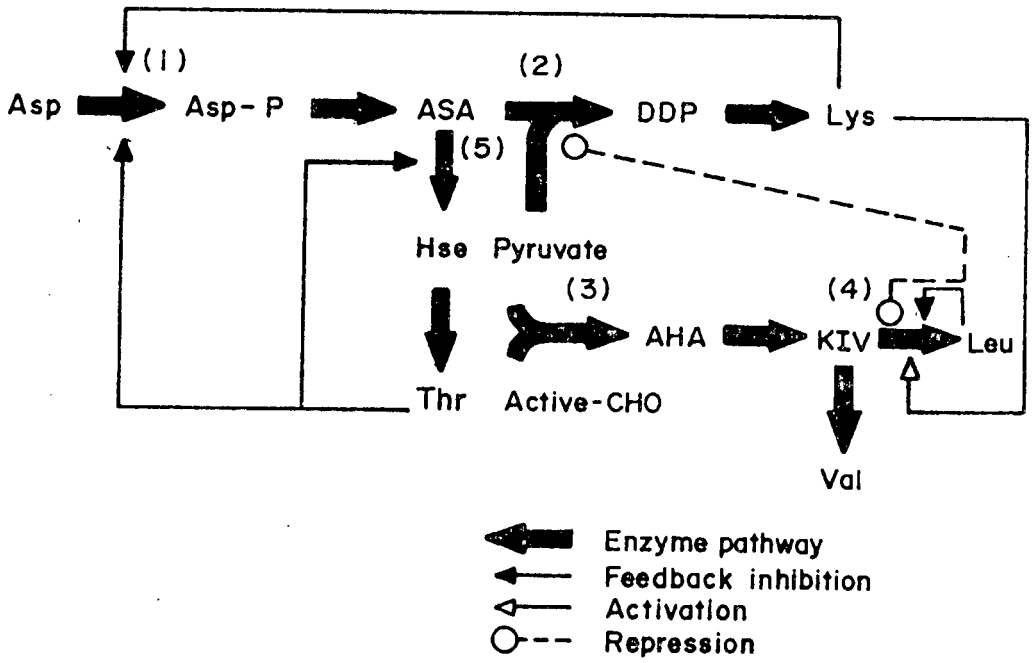


FIG. 4. (1) Aspartate kinase, (2) DDP synthetase, (3) α – acetohydroxy acid synthetase, (4) IPM synthetase, (5) Homoserine dehydrogenase.

Regulation of Lysine and branched chain amino acids biosynthesis in Brevibacterium lactofermentum.

mutants might also be obtained by the induction of revertants from aspartate kinase deficient mutants. Various changes in the enzyme structure might take place and some of them could desensitize the regulatory enzyme.

S-(2-aminoethyl)-L-cysteine, the sulphur analogue of L-lysine, was first investigated by Shiota et al (1958) as a possible inhibitor of lysine utilization due to the striking structural similarity between this synthetic thioesther and lysine. Sano and Shiio (1970) noted that in the case of Brevibacterium flavum S-(2-aminoethyl)-L-cysteine (designated AEC) alone inhibited bacterial growth rather weakly but in the presence of threonine this inhibitory effect was markedly enhanced (fig. 5). This could be explained by the fact that the aspartokinase of this bacterium was inhibited by lysine plus threonine.

AEC resistant mutants were then derived from B.flavum (Sano and Shiio, 1970, 1971). Some of them were found to produce relatively large quantities of L-lysine in minimal medium and were not affected by threonine, thereby differing from the lysine producing auxotrophs. Thus AEC acted as a false feedback inhibitor and the mutation site of these resistant mutants was postulated as being the feedback site of aspartokinase.

Tosaka et al (1978c) tried to obtain mutants of Brevibacterium lactofermentum with an aspartokinase which is genetically desensitized to the feedback inhibition of threonine as well as lysine. They obtained AEC resistant mutants of B.lactofermentum which accumulated large amounts of L-lysine in the culture medium, and found that the aspartokinase of these mutants was not only inhibited by threonine, or threonine plus lysine but also by lysine alone. In order to obtain the desensitization of aspartokinase to feedback inhibition by lysine, mutants were obtained resistant to α-amino-β -hydroxyvaleric acid (AHV) plus lysine. This was a reverse combination of threonine plus AEC in the derivation of AEC resistant mutants. Those AHV resistant mutants which were able to produce increased concentrations of lysine were found to have aspartokinases which were partially desensitized to the feedback inhibition by lysine. This result was supported by the

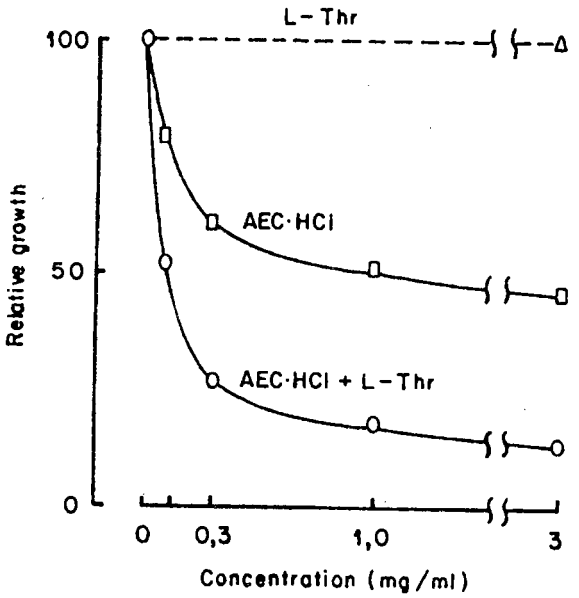


FIG.5. Growth inhibition by 2-aminoethyl-L-Cysteine (AEC) and L-threonine on Brevibacterium flavum. (Hirose etal, 1978).

fact that L-lysine production of AHV and AEC double resistant mutants increased two fold to that of either AHV or AEC resistant mutants. From these results Tosaka et al postulated that a lysine sensitive aspartokinase also exists in the aspartokinase isoenzyme in B.lactofermentum.

Tosaka et al (1978d) were able to obtain high lysine producing mutants of B.lactofermentum by deriving leucine auxotrophs from AEC resistant mutants. They confirmed that leucine auxotrophs derived from AEC resistant mutants of other glutamate producing bacteria, B.saccharolyticum and C.glutamicum also gave high lysine yields.

Tosaka et al (1979) found that a mutant of B.lactofermentum which was resistant to AEC and AHV produced equal quantities of L-threonine and L-lysine. From these observations it was found that the levels of homoserine dehydrogenase (designated HDase) and dihydrodipicolinate synthetase (DDPase) played on important role in the conversion of aspartic semi aldehyde to L-threonine and L-lysine respectively.

DDPase was shown to be inhibited by L-leucine specifically in B.lactofermentum while the formation of HDase was partially inhibited by the addition of methionine. Thus with increased methionine levels threonine production would be reduced and L-lysine production increased. (Fig. 6).

Tosaka et al (1978e) were able to obtain high lysine producing mutants by alanine auxotrophs derived from an AEC resistant mutant of B.lactofermentum. Alanine however, does not directly inhibit the formation of L-lysine. This phenomenon could therefore be explained by the fact that in B.lactofermentum pyruvate and L-aspartate were common precursors in the biosynthesis of L-lysine and L-alanine. (fig. 7). Thus the increased lysine productivity obtained with alanine auxotrophs could result from the reduction of loss of lysine precursors, as pyruvate and L-aspartate would be preferentially converted to L-lysine.

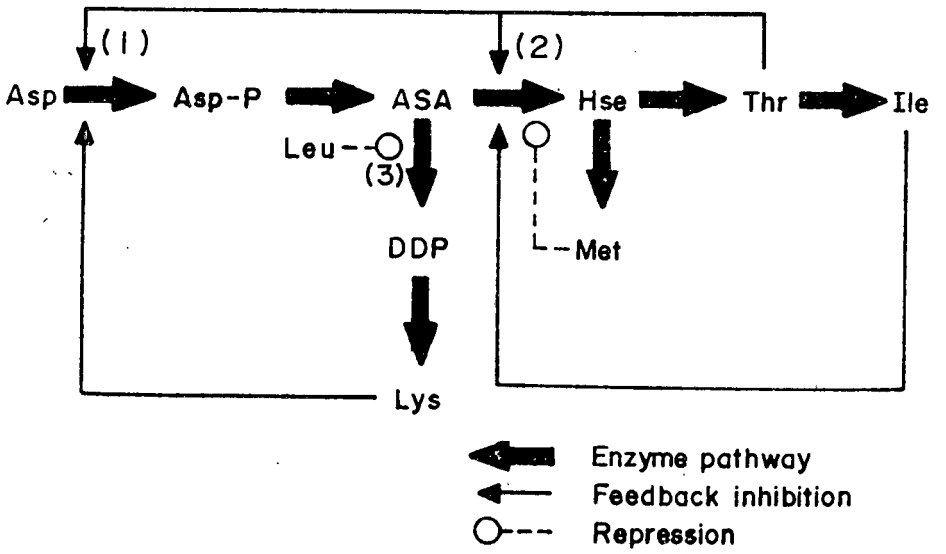


FIG.6. (1) Aspartate kinase, (2) Homoserine dehydrogenase, (3) Dihydrodipicolinate synthetase.

Summary of the regulation of L-lysine and L-threonine biosynthesis in Brevibacterium lactofermentum.

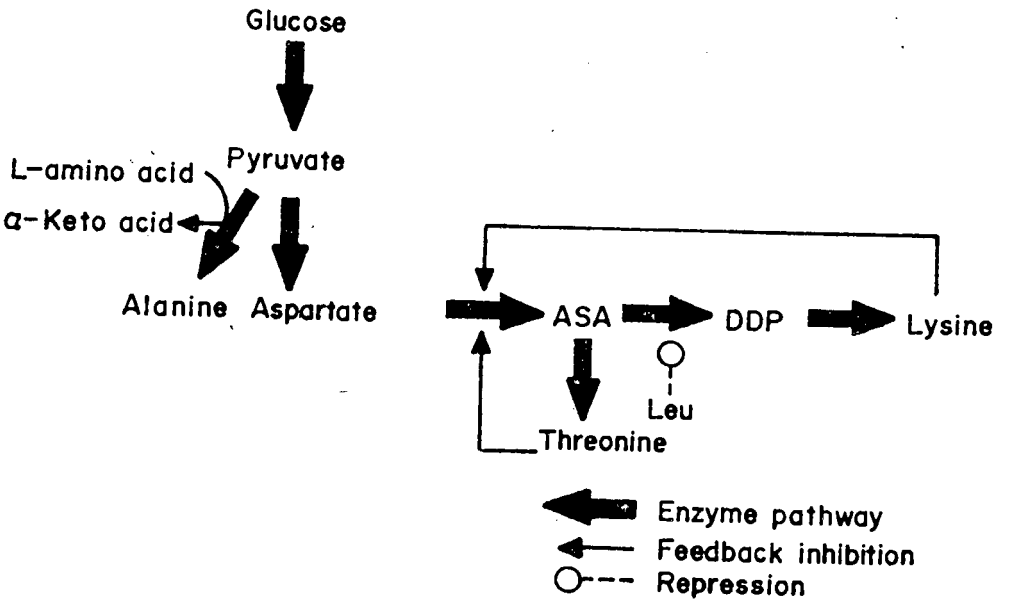


FIG.7. Pathway and regulation of lysine and alanine biosynthesis in Brevibacterium lactofermentum.

The combination of auxotrophy and analogue resistance is a formidible one, resulting in the production of exceptionally high L-lysine yielding mutants. This was further illustrated by Chaudhury et al, (1981) who claimed that when analogue resistance was conferred to a strain of Bacillus subtilis its production of lysine was enhanced.

Further work concerning lysine production by Bacillus species was carried out by Hagino et al, (1981). They looked at thermophilic bacteria as possible lysine producers. The fermentation using Corynebacterium or Brevibacterium is usually carried out at a temperature below 35 °C. A large quantity of cooling water is necessary to keep the fermentation temperature optimal. From the point of reduction of energy consumption it was therefore thought desirable to obtain a bacterium which would be able to assimilate cheap carbon sources such as molasses and starch and also grow at higher temperatures.

They isolated a bacterium able to grow at 46 °C and identified it as Bacillus licheniformis. After mutating it to obtain a homoserine leucine double auxotroph and conferring AEC resistance to it, the bacterium was able to produce relatively large quantities of lysine in a molasses medium at 40 °C.

The utilization of analogue resistant strains as potential high lysine producers was also extended to selected yeast strains. Takenouchi et al, (1977, 1979, 1980) conferred AEC resistance to Candida pelliculosa. The concentration of lysine accumulated by this organism was however ten to fifteen times lower than that accumulated by similar mutants of Brevibacterium and Corynebacterium. Haidaris and Bhattacharijee, (1978) investigated L-lysine production by AEC resistant mutants of Saccharomyces cerevisiae. The amount of lysine produced however was approximately fifty times less than can be obtained with similarly mutated selected bacteria.

Analogue resistant mutants are widely used industrially in the production of various other amino acids. (Kikuchi, 1980). These

include arginine, histidine, proline, isoleucine, valine, leucine and citrulline amongst others.

To conclude, the development of high lysine yielding strains has increased dramatically since the preliminary report published by Kinoshita et al in 1958 concerning the direct production of L-lysine from carbohydrate utilizing a homoserine auxotroph of C.glutamicum.

This increase in lysine production was mainly due to intense investigation of the L-lysine biosynthetic pathway which led to the development of a new range of mutant strains. These comprised auxotrophic mutants, analogue resistant mutants as well as a combination of the two.

It is, however, important to begin with a suitable wild type organism. Ideally it should be a good glutamic acid producer as the glutamate formed can be rapidly transformed into amino acids and other proteinaceous materials. This implies that the strain has potential to synthesize and excrete large amounts of amino acids.

A detailed knowledge of the biosynthetic pathway and more important its regulatory mechanisms is also essential. The regulation of the lysine biosynthetic pathway was found to differ not only between the genera but also from species to species. Regulation was found to be more complex in E.coli than in the glutamate producing bacteria which included Brevibacterium and Corynebacterium.

The overproduction of lysine by auxotrophic mutants was most successful when homoserine leucine or homoserine alanine double auxotrophs were utilized. These double genetic markers not only stabilized the mutants but also facilitated in further overcoming the regulatory control mechanisms of the L-lysine biosynthetic pathway, the exact mechanisms of which were detailed in the previous sections.

Analogue resistance was added to these double auxotrophs causing further increases in lysine production by overcoming the feedback inhibition of lysine on the key enzyme of its biosynthetic pathway.

These mutants proved to be exceptionally high lysine producers with yields of 30 to 40 mg/ml L-lysine HCI, and in some cases even reaching 50 mg/ml. These mutants are used for the industrial production of lysine.

CHAPTER II

A SURVEY OF THE POTENTIAL OF VARIOUS L-LYSINE PRODUCING ORGANISMS

2.1 SUMMARY

Lysine producing bacteria belonging to genera Corynebacterium and Brevibacterium were obtained from the American Type Culture Collection and tested for ability to produce industrially significant yields of lysine. As none of the organisms proved satisfactory, it was decided to initiate a research programme to obtain high lysine producing mutants. Brevibacterium lactofermentum ATCC 13869 was chosen as a suitable wild type strain in view of its good glutamic acid producing capability.

2.2 INTRODUCTION

An extensive survey was conducted on the literature concerning available L-lysine producing organisms. Particular attention was paid to the patent literature as the specific strains mentioned could be obtained from the American Type Culture Collection (designated ATCC) or other such culture collections. The media composition as well as the methods employed were also generally comprehensively documented.

The bacteria listed in the patents as high lysine producers mostly belonged to the genera Corynebacterium and Brevibacterium. The claimed yields varied between 30 mg/mℓ and 54 mg/mℓ L-lysine.HCl.

As mentioned in section 1.3 the most common mutants which tended to give the highest yields of L-lysine were those which were homoserine, leucine double auxotrophs as well as being resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine. Three strains were obtained from the ATCC which, according to their patents, were high lysine producers. The yields claimed for these particular microorganisms varied between 37 mg/mℓ and 54 mg/mℓ L-lysine.HCl. These strains

were then tested for L-lysine production according to their patent specifications.

It is important to determine the glutamic acid production potential of wild type strains intended as lysine producers. Kinoshita et al (1957) recognized the important link between the glutamic dehydrogenase system and the metabolism of amino acids and carbohydrates, as the glutamate formed can be rapidly transformed into various other amino acids and proteinaceous materials. Thus in a search for potential high lysine yielding microorganisms it is important that the glutamic acid producing ability of the wild type strains intended as possible L-lysine producers should be as high as possible.

2.3 METHODS

2.3.1 Patent Literature Survey

A comprehensive collection of the patent literature concerning lysine production by various microorganisms is listed in Appendix A.

2.3.2 Media, buffers and routine reagents

These are detailed in Appendix B.

2.3.3 Strains utilized in subsequent experiments

The following strains were obtained from the American Type Culture Collection Catalogue of Strains (Gherna et al, 1978.)

(i) Corynebacterium glutamicum
ATCC 21513
Genotype : homoserine$^-$ leucine$^-$ penicillin^R.

(ii) Brevibacterium lactofermentum
ATCC 21086
Genotype : Isoleucine$^-$ threonine$^-$ valine$^-$

(iii) Corynebacterium glutamicum
ATCC 21526
Genotype : homoserine$^-$ leucine$^-$ S-(2-aminoethyl)-L-cysteine^R.

(iv) Corynebacterium glutamicum
ATCC 13032
Genotype : wild type.

(v) Brevibacterium lactofermentum
ATCC 13869
Genotype : wild type

(vi) Brevibacterium flavum
ATCC 14067
Genotype : wild type

2.3.4 L-lysine fermentations utilizing the following strains respectively : C.glutamicum ATCC 21526; C.glutamicum ATCC 21513 and B.lactofermentum ATCC 21086

C.glutamicum ATCC 21526 was tested for its ability to produce lysine according to the method described by Nakayama and Araki (1973). A loopful of bacteria from a 24 h nutrient agar slant of ATCC 21526 was used to inoculate 20,0 mℓ of seed medium in a 250 mℓ Erlenmeyer flask. This was incubated at 30 °C on an orbital shaker at 170 rpm for 24 h. Five percent of the seed medium was used to inoculate 20,0 mℓ of fermentation medium in a 250 mℓ Erlenmeyer flask. The fermentation was carried out at 30 °C on an orbital shaker for 4 days. An uninoculated flask of fermentation medium was included as a control. After 4 days an aliquat was removed, suitably diluted and plated onto nutrient agar plates in order to check for growth. The fermentation medium was centrifuged at 10 000 rpm for 15 min, and the supernatant assayed for the presence of L-lysine.

C.glutamicum ATCC 21513 was tested for lysine production following the method as described by Inuzuka and Hamada (1976). This was essentially the same as described above, except that the fermentation medium was assayed for the presence of L-lysine after 48 h and the composition of the media also differed (Appendix B).

B.lactofermentum ATCC 21086 was tested for L-lysine production according to the method described by Kubota et al (1970). This method differed from that employed for ATCC 21526 and ATCC 21513 in that the fermentation medium was inoculated directly from a 24 h nutrient agar slant, thus no seed medium was used. The fermentation was conducted over three days and the composition of the medium also differed (Appendix B.)

2.3.5 Methods for L-lysine determination

Quantitative determination of L-lysine was done according to the method of Chinard (1952). The results were verified using a Technicon TSM amino acid analyser (Ertingshausen and Adler, 1970).

2.3.6 Glutamic acid production by C.glutamicum ATCC 13032; B.lactofermentum ATCC 13869 and B.flavum ATCC 14067 respectively

The method for glutamic acid production as described by Otsuka et al (1965) was followed. Fifty mℓ of basal medium was added to 250 mℓ Erlenmeyer flasks. Concentrations of biotin ranging from 0,5 µg/ℓ to 2,0 µg/ℓ were added to each flask from a filter sterilized stock solution. Each flask was inoculated by flooding a 24 h nutrient agar slant of each of the above mentioned bacteria respectively with 7,0 mℓ of 0,1 M sodium phosphate buffer pH 7,0. A 0,2 mℓ aliquot of each bacterial suspension was inoculated into each flask. An uninoculated blank was included. The flasks were left on an orbital shaker at 170 rpm and 30 °C for 72 h. At 8 and 16 hour time intervals, 7,0 mℓ aliquats were taken. The pH was noted and growth determined by turbidimetric measurement of the suitably diluted sample at 562 nm using a Pye Unicam SP6-550 U.V. spectrophotometer. Samples were then centrifuged at 10 000 rpm for 15 min and the supernatants assayed for glutamic acid presence.

2.3.7 Method for determining glutamic acid

Samples were analysed for glutamic acid using a Technicon TSM amino acid analyser (Ertingshausen and Adler, 1970.)

2.4 RESULTS AND DISCUSSION

2.4.1 L-Lysine concentrations obtained from ATCC cultures

C.glutamicum ATCC 21526, C.glutamicum ATCC 21513 and B.lactofermentum ATCC 21086 produced respectively: 0,5 mg/mℓ L-lysine.HCI ($2,1 \times 10^8$ cells/mℓ); 2,1 mg/mℓ L-lysine.HCI ($3,8 \times 10^8$ cells/mℓ); and 1,3 mg/mℓ L-lysine.HCI ($4,0 \times 10^7$ cells/mℓ). These three cultures obtained from the ATCC and tested for their abilities to produce lysine according to their patent specifications proved disappointing. The concentrations of L-lysine.HCI produced were twenty to a hundred times lower than those specified in the respective patents.

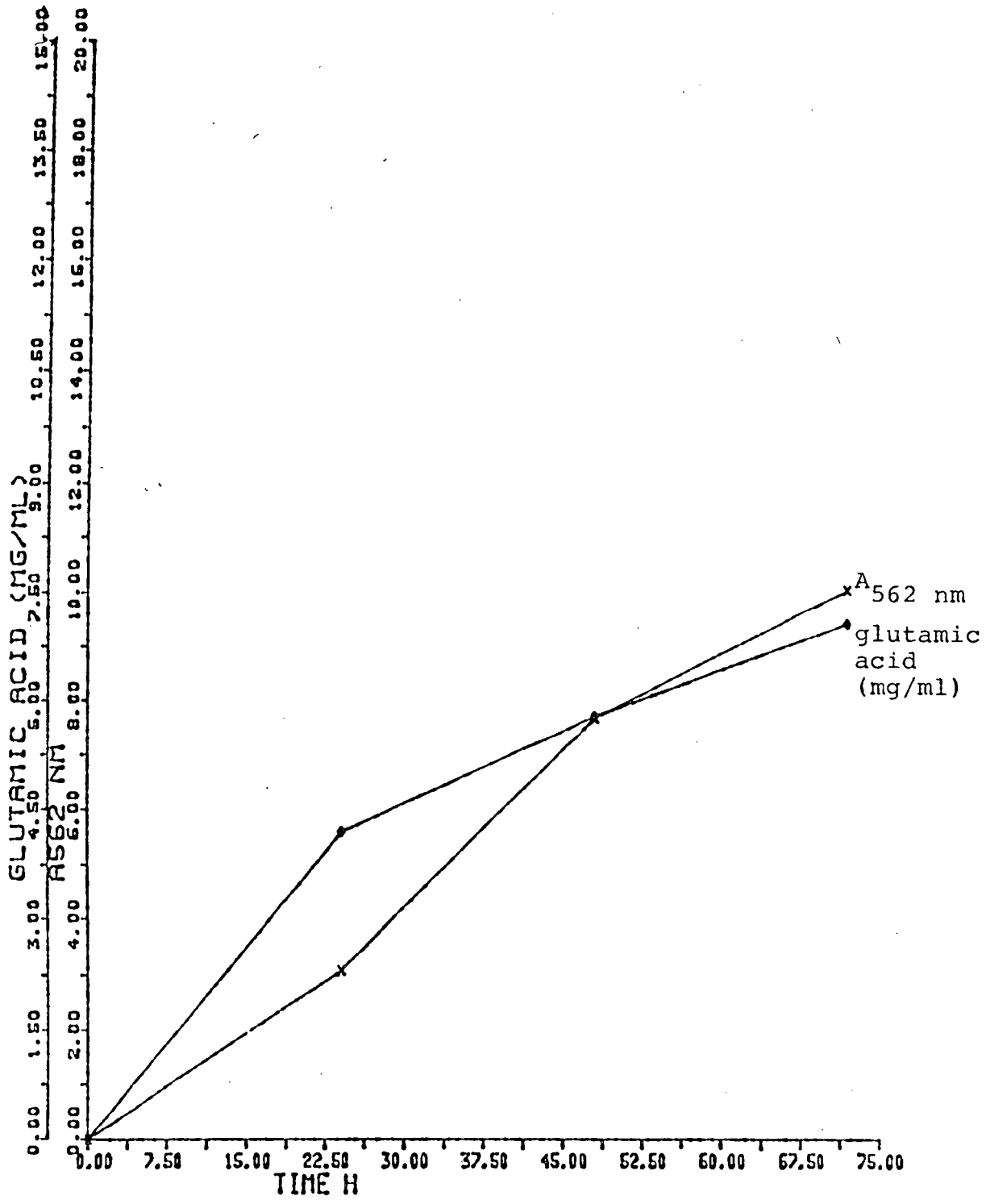
Although the production of L-lysine by these organisms could possibly have been increased through optimization procedures, it was decided that as the yields were so much lower than specified a mutational research programme would be initiated to find high lysine producing microorganisms.

2.4.2 Growth and glutamic acid production of C.glutamicum ATCC 13032; B.flavum ATCC 14067 and B.lactofermentum ATCC 13869

It is important to determine the glutamic acid production potential of organisms intended as lysine producers. (Section 2.1).

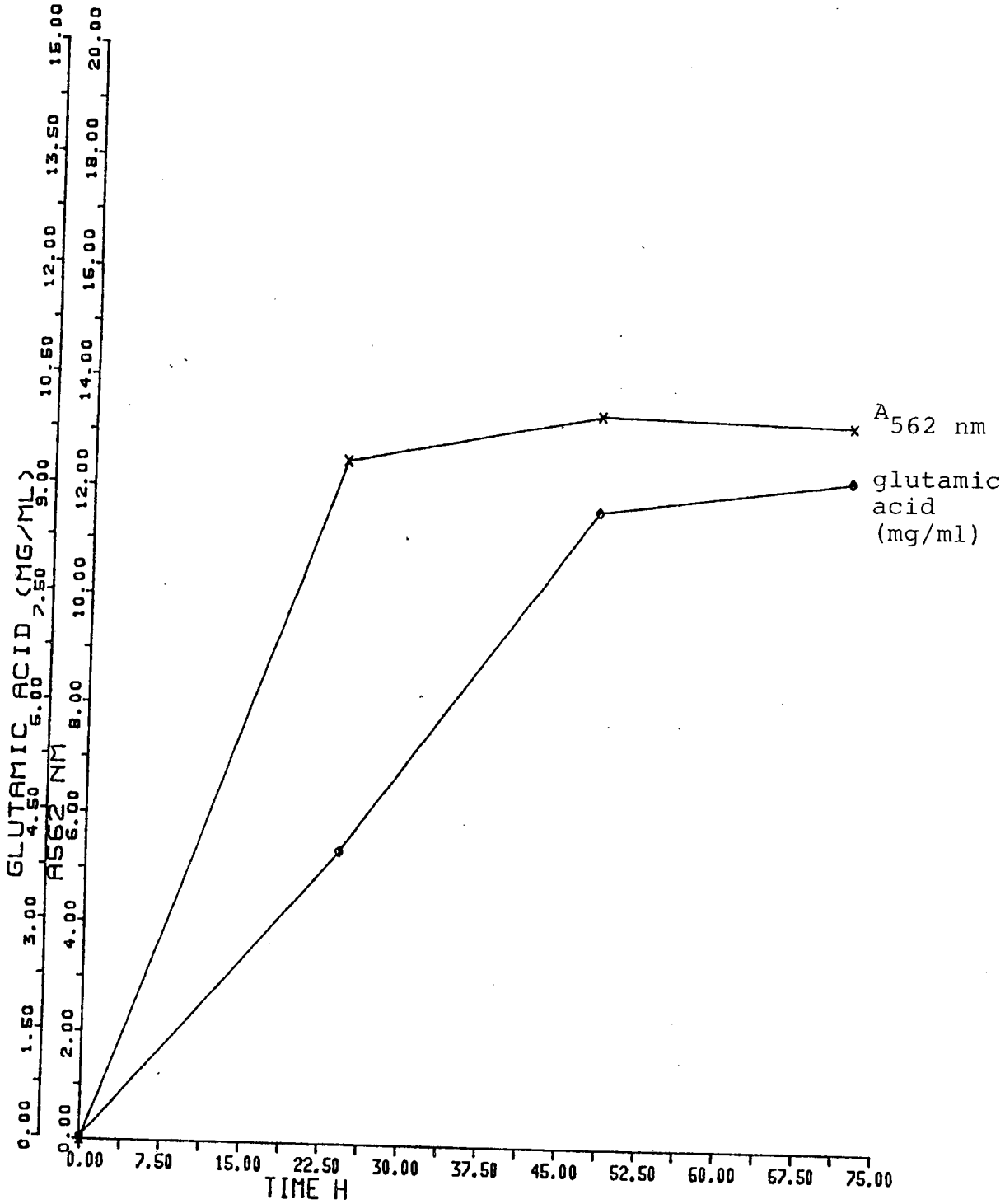
After examining the relevant literature, three wild type strains were obtained from the ATCC as potential high lysine producing microorganisms. They were members of the genera Corynebacterium and Brevibacterium and were all natural biotin auxotrophs.

The growth and glutamic acid production of C.glutamicum ATCC 13032, B.flavum ATCC 14067 and B.lactofermentum ATCC 13869 are shown in figures 8, 9 and 10 at their respective optimum biotin concentrations conducive for high levels of glutamate production. After growth for 72h they produced 7,1 mg/mℓ (48 µmol/mℓ); 9,2 mg/mℓ (62,3 µmol/mℓ) and 13,4 mg/mℓ (91,1 µmol/mℓ) glutamic acid respectively.



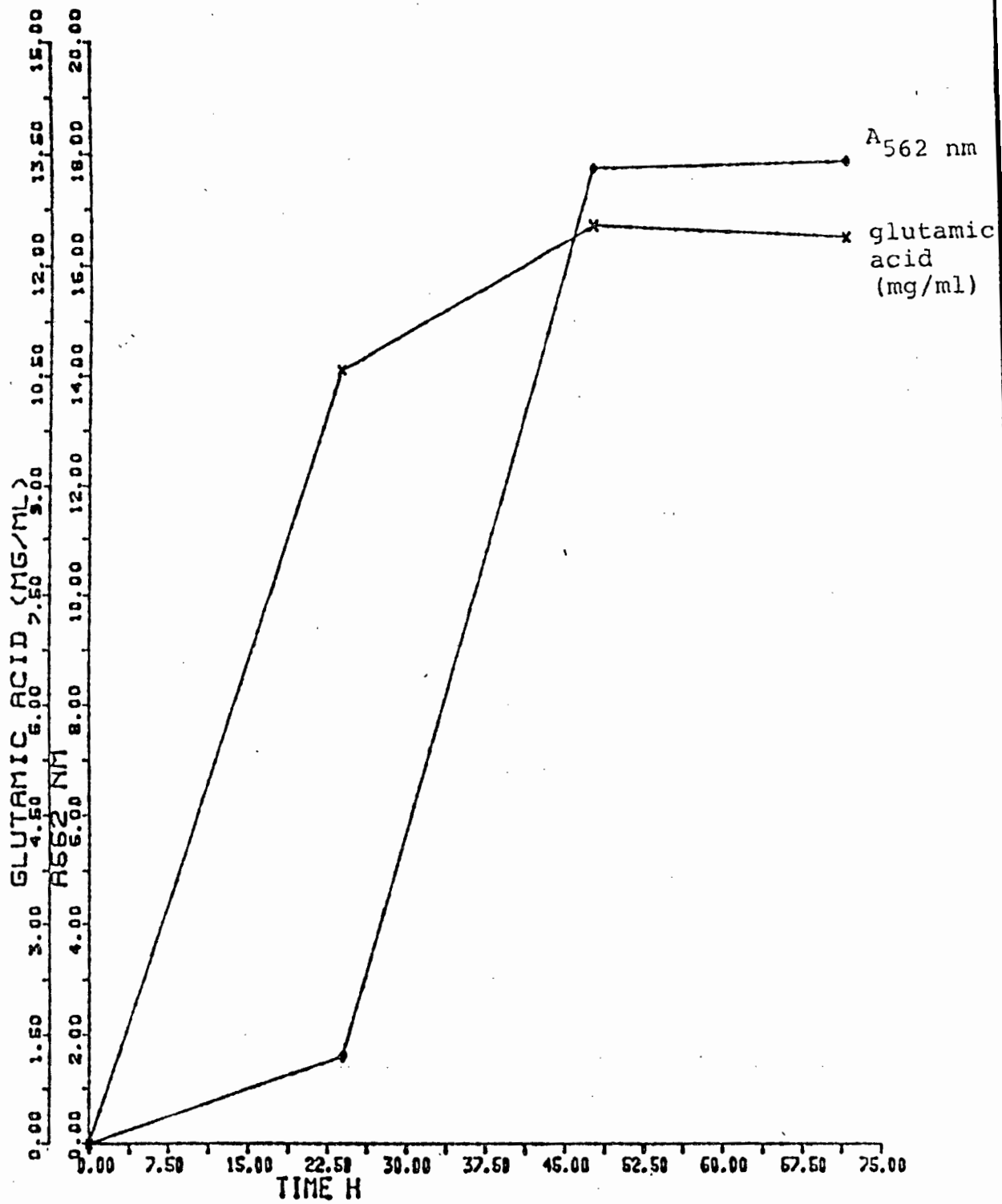
Production of glutamic acid and growth by C. glutamicum (0,5 µg/ℓ biotin)

FIG. 8



Production of glutamic acid and growth by B. flavum (2,0 µg/ℓ biotin)

FIG.9



Production of glutamic acid and growth by B. lactofermentum (1,0 µg/ℓ biotin)

FIG. 10

The most promising strain selected was B.lactofermentum ATCC 13869, as it was able to produce 13,4 mg/mℓ glutamic acid which is a yield of 41% in relation to the glucose supplied. All industrially important strains can produce from 30 g to 50 g of glutamic acid from 100 g of glucose in 1 litre of medium.

These microorganisms were found to be extremely sensitive to even very small concentrations of biotin. The addition of low levels of biotin to the medium was able to markedly stimulate or adversly affect not only growth, but also their glutamic acid producing abilities.

It was noted particularly with B.lactofermentum that most of the glutamic acid was produced during the early stationary phase of growth. These findings agree with the results obtained by Otsuka et al (1965).

CHAPTER III

AUXOTROPH SELECTION

3.1 SUMMARY

Homoserine, leucine and homoserine leucine auxotrophs were obtained from B.lactofermentum ATCC 13869 and C.glutamicum ATCC 13032. They were screened for their ability to produce lysine. BL 1136 hse^- and B57 $hse^- leu^-$ isolated from B.lactofermentum ATCC 13869 were chosen as the two most promising candidates for further mutational study. They gave respectively a 24 fold and 37 fold increase in lysine production over the parent strain.

3.2 INTRODUCTION

It was noted in section 2.4 that of the two Brevibacterium species, B.lactofermentum produced the highest concentration of glutamic acid when 1 µg/ℓ biotin was used to supplement the medium. It was therefore decided to use this organism as well as a member of the Corynebacterium genus, C.glutamicum in future mutational studies involving the selection of a high lysine producing mutant.

The fact that over production of most amino acids including lysine by wild type microorganisms is small, is due to the efficient regulatory mechanisms in the biosynthetic pathways. Auxotrophic mutants represent one method of artificially disorganizing such regulations. In fig 6 section 1.3.3 the regulation of L-Lysine biosynthesis in B.lactofermentum is illustrated. It can be seen that threonine and lysine exert feedback inhibition on aspartate kinase a key enzyme for the synthesis of lysine. L-leucine exerts feedback inhibition and repression on dihydrodipicolinate synthetase. Thus a bacterium unable to produce threonine and methionine (or homoserine) would be suitable for lysine production, there being no feedback inhibition or repression as threonine and methionine (or homoserine) could be added at suboptimal amounts just sufficient for growth. Similarly a bacterium unable to synthesize leucine would give higher lysine yields as the

feedback inhibition and repression on the dihydrodipicolinate synthetase enzyme would be absent. In order to overcome the feedback inhibition caused by a high concentration of lysine, an analogue of lysine was employed. This will be delt with in greater detail in chapter IV.

Further factors in favour of Brevibacterium and Corynebacterium as potential high lysine producers are that no lysine degrading activity is found and there is no permeability barrier against lysine in either genus.

To obtain auxotrophs from B.lactofermentum and C.glutamicum, the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (designated NTG) was employed as the mutagen. NTG was chosen as it is an extremely efficient mutagenic agent and has been shown in Escherichia coli to induce at least one mutation per treated cell under conditions permitting over fifty per cent survival, while auxotroph yields well in excess of ten per cent of the treated population can be achieved. (Adelberg et al, 1965).

A series of survival curves were done utilizing different concentrations of NTG and exposing the bacteria to the NTG for varying time intervals. In this way the optimum conditions for mutagenesis by NTG could be established.

The isolation of auxotrophic mutants was further facilitated by the introduction of a semi-selective method based on the mode of action of penicillin (Davis, 1948; Lederberg and Zinder, 1948) which rapidly kills growing bacteria but is innocuous to those which are not growing.

By utilizing these techniques several homoserine auxotrophs, leucine auxotrophs and homoserine leucine double auxotrophs were obtained from B.lactofermentum and C.glutamicum. These were then tested for their lysine producing abilities.

3.3 METHODS

3.3.1 Media, buffers and routine reagents

These are detailed in the appendix.

3.3.2 Selection for homoserine and leucine auxotrophs and for homoserine, leucine double auxotrophs of C.glutamicum ATCC 13032 and B.lactofermentum ATCC 13869

A series of NTG survival curves were used to determine the optimum concentration of NTG which would yield the greatest number of mutants.

Cells of C.glutamicum ATCC 13032 and B.lactofermentum ATCC 13869 respectively were harvested in the late logarithmic growth phase, washed with 0,05 M sodium phosphate buffer (pH 8,0) and resuspended in an equal volume of the same buffer to which 150 µg/mℓ NTG had been added. After incubation for 20 min. at 30 °C the bacterial populations respectively decreased by approximately 90%. The penicillin selection technique of Davis (1948), Gorini and Kaufman (1960) was followed. The suspension was centrifuged at 5000 rpm for 10 min. washed with minimal medium (Tosaka et al, 1978) and resuspended in minimal medium plus homoserine (Sano and Shiio, 1971) and leucine (Nakayama and Araki, 1973). After incubation at 30 °C on an orbital shaker for 24 h the bacteria were again harvested, washed with minimal medium and resuspended in an equal volume of minimal medium. The bacterial suspension was again left on the orbital shaker at 30 °C until log phase was reached. Penicillin was added at a concentration of 10 units/mℓ (Nakayama et al, 1961) and the culture left to incubate for a further 16 h. The cells were harvested, resuspended in the original volume of minimal medium, diluted and plated onto nutrient agar plates. The colonies were then transferred to nutrient agar and minimal medium plates and scored for growth. Colonies which failed to grow on minimal medium plates were classified as possible auxotrophs, and were tested for homoserine and leucine auxotrophy and homoserine leucine double auxotrophs.

3.3.3 Screening auxotrophs for L-Lysine production

A loopfull of bacteria from a 24 h nutrient agar slant of each of the mutants to be screened was inoculated into 30 mℓ of seed medium (Nakayama et al, 1961) in a 250 mℓ Erlenmeyer flask. The flasks were incubated on an orbital shaker at 170 rpm for 24 h at 30 °C. Ten per cent of the seed medium was used to inoculate 30 mℓ of fermentation medium (Nakayama et al, 1961) in 250 mℓ Erlenmeyer flasks. The fermentation was conducted at 30 °C on an orbital shaker at 170 rpm. After four days the medium was centrifuged at 10 000 rpm for 15 min. and the supernatant assayed for L-lysine. An uninoculated flask was used to determine the initial lysine value in the medium. C.glutamicum ATCC 21513 or B.lactofermentum ATCC 21086 was included with each screening procedure as a control.

3.3.4 Methods of L-lysine determination

Quantitative determination of L-lysine was initially done according to the method of Chinard (1952) and then by the method of Gaillardin et al (1975). Both these methods were found to give variable results and were later abandoned in favour of paper chromatography (Walczak and Oberman, 1980). Whenever a possible high lysine producing mutant was detected by any of the above methods the results were verified using a Technicon TSM amino acid analyser following the method of Ertingshausen and Adler (1970).

3.3.4.1 Descending paper chromatography. Sample preparation for chromatography

In order to determine the concentration of lysine present in various samples a L-lysine standard curve was first determined. L-lysine standards of between 0,5 mg/mℓ and 4 mg/mℓ were made with distilled water, and applied to the chromatogram. Each sample to be analysed was diluted appropriately with distilled water before application.

Chromatogram preparation and running conditions

Whatman No. 1 chromatography paper of 20 cm width and 60 cm length was used. Ten µℓ of each suitably diluted sample and standard was applied with a micropet ten µℓ disposable pipette at 2 cm intervals 7 cm from the end of the chromatogram. The chromatogram was allowed to equilibrate for approximately 30 min. and then run for 16 to 20 h at room temperature. The chromatograms were run in glass chromatography tanks. The solvent mixture was n-butanol, acetic acid and distilled water in the ratio of 12:3:5 (^{v}/v).

Detection of L-lysine

L-Lysine was detected by dipping the dried chromatogram into a solution of 0,2% (^{m}/v) ninhydrin in acetone and then leaving the chromatogram at 100 °C ± 10 °C for 5 min.

Quantitative determination of L-lysine

The ratio of the distance travelled by the compounds to the distance travelled by the solvent front from the original spot on the paper sheet was measured. (R_f value). Each spot was cut out and left to elute for 1 h in a 5,0 mℓ mixture of methanol, distilled water and $CuSO_4.5H_2O$ (Appendix B). The absorbance was measured at 516 nm on a Unicam U.V. spectrophotometer using a reagent blank. The concentration of L-lysine in the samples was then calculated using the L-lysine standard curve. However the graph of the lysine standards plotted against abosrbance was not linear over the whole range. It was therefore decided to include two standards from the most linear part of the graph with each chromatogram. From these standards, namely 0,75 mg/mℓ and 1,5 mg/mℓ L-lysine.HCl a curve could be drawn and the lysine concentrations in each sample calculated.

3.4 RESULTS AND DISCUSSION

3.4.1 NTG mutagenesis of C.glutamicum ATCC 13032 and B.lactofermentum ATCC 13869

A total of 571 auxotrophs were obtained from 1550 colonies of C.glutamicum screened. A 37% auxotroph rate was achieved. This is in agreement with the results given by Nakayama et al (1961b) who used the penicillin selection technique and found that 10u/mℓ penicillin gave an optimum auxotroph rate of 39,2%. From these mutants 2 hse$^-$, 8 leu$^-$ and 3 hse$^-$leu$^-$ auxotrophs were obtained.

In the case of B.lactofermentum, 412 auxotrophs were obtained from 1900 colonies screened. A 22% auxotroph rate was achieved. From these mutants 17 hse$^-$, 25 leu$^-$ and 4 hse$^-$leu$^-$ auxotrophs were obtained.

3.4.2 Screening mutants obtained from C.glutamicum ATCC 13032 and B.lactofermentum ATCC 13869 for lysine production

Of the mutants isolated from C.glutamicum ATCC 13032, 46% gave more than a 10 fold increase in lysine production over the wild type parent strain, while the highest lysine producer obtained gave an 83 fold increase over the parent strain (table 3).

From B.lactofermentum ATCC 13869 13,6% of the mutants obtained gave more than a 10 fold increase in lysine production over the wild type parent strain. The best lysine producer isolated gave a 37 fold increase over the parent strain (table 4).

It was found that mutants obtained from C.glutamicum with the leu$^-$ genotype tended to produce higher concentrations of lysine than those with the hse$^-$ or hse$^-$leu$^-$ genotypes. While screening the mutants of B.lactofermentum it was found that one of the hse$^-$leu$^-$ double auxotrophs followed by certain of the hse$^-$ auxotrophs produced the

Mutant Number	L-lysine.HCl (mg/mℓ)	Genotype
C 321	2,8	hse^-
C 2	0,4	hse^-
C 167	8,3	leu^-
C 279	6,7	leu^-
C 426	6,3	leu^-
C 429	0,9	leu^-
C 108	0,5	leu^-
C 398	0,4	leu^-
C 48	0,3	leu^-
C 427	trace	leu^-
C 1	3,8	hse^-leu^-
C 14	3,5	hse^-leu^-
C 31	0,4	hse^-leu^-
ATCC 13032	0,1	wild type
ATCC 21513	3,7	$hse^-leu^-Pen^R$

Amount of lysine produced by mutants obtained from C.glutamicum ATCC 13032

Table 3

Mutant Number	L-lysine.HCl (mg/mℓ)	Genotype
BL 608	11,3	hse$^-$
BL 493	9,9	hse$^-$
BL 1136	9,7	hse$^-$
B 691	8,6	hse$^-$
BL 903	5,1	hse$^-$
BL 1792	3,5	hse$^-$
BL 209	2,6	hse$^-$
B 725	2,0	hse$^-$
BL 726	1,6	hse$^-$
BL 66	0,9	hse$^-$
B 1240	0,4	hse$^-$
BL 702	0,2	hse$^-$
BL 599	0,1	hse$^-$
BL 394	0,1	hse$^-$
B 1154	trace	hse$^-$
B 1468	trace	hse$^-$
BL 37	2,8	leu$^-$
BL 619	1,1	leu$^-$
BL 1655	1,0	leu$^-$
BL 1450	0,8	leu$^-$
BL 130	0,7	leu$^-$
BL 571	0,6	leu$^-$
BL 93	0,5	leu$^-$
B 1201	0,5	leu$^-$
BL 494	0,4	leu$^-$
BL 20	0,4	leu$^-$
B 1215	0,4	leu$^-$
BL 219	0,3	leu$^-$
BL 527	0,3	leu$^-$
BL 777	0,3	leu$^-$
B 1257	0,3	leu$^-$
BL 1391	0,2	leu$^-$

Table 4 (cont.)

Mutant Number	L-lysine.HCl (mg/mℓ)	Genotype
B 827	0,2	leu^-
B 1241	0,2	leu^-
B 1250	0,2	leu^-
BL 175	trace	leu^-
BL 496	trace	leu^-
BL 222	0,0	leu^-
B 1110	0,0	leu^-
B 1221	0,0	leu^-
B 57	14,9	$hse^- leu^-$
B 1098	0,2	$hse^- leu^-$
B 1528	trace	$hse^- leu^-$
BL 602	0,0	$hse^- leu^-$
ATCC 13869	0,4	wild type
ATCC 21086	4,9	$Thr^- Val^- Isoleu^-$

Amount of lysine produced by mutants obtained from B.lactofermentum ATCC 13869

Table 4

highest concentration of lysine. Those with the leu$^-$ genotype tended to produce relatively little lysine.

Of the many auxotrophs screened the best mutant obtained was B57 hse$^-$leu$^-$ which gave 14,9 mg/mℓ L-lysine.HCI on the screening medium. Another potentially significant mutant was the homoserine auxotroph BL 1136 which gave 9,7 mg/mℓ L-lysine.HCI on the same screening medium. Although not the highest lysine producer amung the homoserine auxotrophs, it was discovered first and was later found to respond well to optimization.

The auxotrophic mutants obtained from the ATCC were also re-tested on the screening medium for comparative purposes, as their poor performance on molasses medium could have been attributed to the quality of the molasses available. However, their yields of lysine, although better than those obtained on the patent specified media were still low especially when compared to some of the mutants obtained.

CHAPTER IV

ISOLATION OF ANALOGUE RESISTANT MUTANTS

4.1 SUMMARY

Analogue resistant mutants were isolated from B.lactofermentum ATCC 13869, BL 1136 hse$^-$ and B57 hse$^-$leu$^-$. These were found to be unstable and reverted. The screening medium was improved and D14 was isolated from B57 as a potential AEC^R mutant. D14 was able to produce 19,2 mg/mℓ L-lysine.HCl which was approximately a 20% increase over B57.

The levels of aspartate kinase in B.lactofermentum ATCC 13869, BL 1136, B57 and D14 were investigated. The aspartate kinase specific activity of D14 was found to be 240% higher than that of the wild type. This could account for its high lysine producing capability as the aspartate kinase of D14 was not found resistant to repression by L-threonine and L-lysine.

Inhibition studies carried out on the aspartate kinase of B.lactofermentum ATCC 13869, BL 1136, B57 and D14 indicate a possible novel regulation system occurring in the lysine biosynthetic pathway of this organism.

4.2 INTRODUCTION

In Chapter III an attempt was made to overcome the efficient regulatory mechanisms associated with lysine production by selecting for certain auxotrophic mutants. However, through feedback inhibition, lysine is able to regulate its own production. Thus in order to overcome this control mechanism a second technique was utilized involving regulatory mutants. Regulatory mutants possess a feedback-insensitive key enzyme and can be expected to accumulate the related amino acid in large amounts.

A classic example of the application of regulatory mutants to amino acid fermentation was in the field of lysine production. This was because the regulation of L-lysine biosynthesis in Brevibacterium was relatively simple as only aspartate kinase was sensitive to feedback inhibition by lysine as opposed to the L-lysine biosynthetic pathway found in E.coli where L-lysine exerts feedback inhibition and repression at a number of sights.

Regulatory mutants were obtained by the isolation of L-lysine analogue resistant mutants whose growth was not inhibited by a L-lysine analogue, which behaved as a feedback inhibitor on aspartate kinase and which did not allow growth of the parent strain. Some of the mutants resistant to the analogue were expected to be regulatory mutants with aspartate kinase desensitized to concerted feedback inhibition.

S-(2-aminoethyl)-L-cysteine (AEC) is the most common L-lysine analogue employed in searching for L-lysine analogue resistant mutants. A successful AEC resistant regulatory mutant usually exhibits the following characteristics:

(i) Its growth is no longer influenced by AEC which inhibited growth of the parent strain with false feedback inhibition.

(ii) It produces large amounts of L-lysine.

(iii) L-lysine production by the mutant is not restricted by exogenous L-threonine and L-lysine.

(iv) Activity of its aspartate kinase is not inhibited by L-threonine and L-lysine.

Tosaka and Takinami (1978), Tosaka et al (1978b), Tosaka et al (1978c) found that the concentrations of L-lysine produced by auxotrophic mutants was greatly increased when analogue resistance was conferred

on them, and conversely when auxotrophic mutants were obtained from analogue resistant mutants the concentrations of L-lysine were similarly increased.

Sano and Shiio (1970), Qi (1981) showed that even wild type strains are able to give a substantially increased L-lysine production capability when analogue resistance was conferred on them.

It was therefore decided that the concentration of lysine produced by the homoserine auxotroph BL 1136 and in particular the high yielding homoserine leucine double auxotroph B57 might be greatly increased if analogue resistant mutants were isolated from them.

An attempt was also made to isolate analogue resistant mutants from the wild type parent strain B.lactofermentum ATCC 13869 so as to determine its potential as a L-lysine producer with a view to then mutating it further to obtain auxotrophic mutants.

However, reversion was found to be a problem among most of the potential AEC resistant high lysine producing mutants obtained. It was thought possible to protect these mutants further by exposing them to a different mutagen and re-selecting AEC resistant mutants. The analogue resistant phenotype might then be enforced preventing reversion and possibly even increasing the production of L-lysine.

Alternatively if B57 were exposed to NTG treatment, and analogue resistant mutants selected from agar plates in which other lysine analogues were incorporated in lieu of AEC then it might facilitate the selection of a stable analogue resistant mutant. The L-lysine analogues utilized were L-lysine hydroxamate (LH) and N-ε-methyl-L-lysine.HCl (NEM) (Sano and Takayasu, 1980).

The mutagen used to enforce the AEC resistant genotype of D14 was ethyl methane sulphonate (EMS). It is a member of the group of monofunctional alkylating agents. The mutations induced by EMS are similar to those induced by UV light in that they are randomly

distributed over the genome. Guerola and Cerdá-Olmedo (1975) and Plachy (1968) found that when isolating auxotrophic mutants from a strain of Corynebacterium, EMS had a high mutagenic effect even when its lethal effect was low.

EMS appears to act specifically on guanine at the N-7 position labilizing the deoxyriboside linkage so that 7-alkyl guanine is released from the DNA. (Hayes, 1970). This offers a plausible explanation of its mutagenic effect since the missing guanines might be replaced by any one of four bases resulting in a permanent change in base sequence.

It was decided to examine the aspartate kinase activities of the mutant strains as well as of the wild type parent strain with a view to determining whether the regulation by L-threonine and L-lysine in vitro differed between the mutants. This could possibly illustrate the effect of the different genotypes on the lysine biosynthetic pathway of the various mutants and specifically that of D14 hse$^-$ leu$^-$ AEC^R.

4.3 METHODS

4.3.1 Media buffers and routine reagents

These are detailed in the appendix.

4.3.2 Isolation of analogue resistant mutants from B.lactofermentum ATCC 13869, BL 1136 and B57

The method followed was based on that of Tosaka et al (1978c). Aliquots (50 mℓ) of medium I broth were added to 500 mℓ Erlenmeyer flasks and inoculated with a loopful of cells from 48 h medium I slants of B.lactofermentum ATCC 13869, BL 1136 or B57. The flasks were shaken at 30 °C and 170 rpm for 24 h. Each culture was diluted 10 X into fresh medium I broth and incubated further until late logarithmic growth phase was reached (approximately 3 h). An aliquot from each culture was removed and centrifuged at 5 000 rpm for 15 min. The cells were then washed twice with 0,1 M sodium phosphate buffer pH 7,0 and resuspended in the same volume of buffer. NTG (250 ug/mℓ) was added and the suspensions left at 30 °C in a shaking water bath for 25 min. The bacteria were then harvested by centrifuging at 5 000 rpm for 15 min. and washed twice with the 0,1 M sodium phosphate buffer pH 7,0. They were resuspended in the same volume of buffer, diluted and plated onto medium 2 agar plates supplemented with 4 mM AEC plus 4 mM L-threonine. However, in the case of mutants derived from BL 1136, homoserine was added as well as AEC and threonine, while for those mutants obtained from B57 leucine as well as homoserine was added to the medium. These plates were left to incubate at 30 °C for 4 to 7 days. The colonies growing on the plates were then screened for high lysine producers.

Further attempts were made to obtain stable AEC resistant mutants from B57. The method followed was essentially the same as described above except that the concentration of NTG used was 200 ug/mℓ and 2,5 mg/mℓ AEC and L-threonine respectively was used to supplement the medium 2 agar plates.

4.3.3 Isolation of analogue resistant mutants from B57 utilizing the lysine analogues LH, NEM and AEC

The method as set out in section 4.2.3 was followed except that the medium 2 agar plates contained 4 mM L-lysine hydroxamate or 4 mM N-ε-methyl-L-lysine.HCl. In addition 4 mM AEC plates were also utilized. To each of the three different sets of agar plates supplemented with the various lysine analogues, 4 mM L-threonine was also added. The plates were incubated for 4 to 6 days at 30 °C.

4.3.4 Isolation of analogue resistant mutants from D14 utilizing the mutagen EMS

D14 hse$^-$ leu$^-$ AEC^R derived from B57 was used as the parent strain for the isolation of further analogue resistant mutants, utilizing the mutagen EMS.

A series of EMS survival curves were carried out so as to determine the optimum concentration of EMS required in order to obtain a high percentage of mutants.

A logarithmic phase bacterial suspension was obtained. An aliquot of this suspension was centrifuged, washed and the bacteria resuspended in 0,1 M phosphate buffer pH 7,0. Four percent (^{v}/v) EMS was added and the suspension was left shaking at 30 °C in a waterbath for 60 min. giving a survival of approximately 10%. The bacterial suspension was then centrifuged at 6 000 rpm for 10 min, suitably diluted with 0,1 M phosphate buffer and plated onto medium 2 plates supplemented with 4 mM AEC and 4 mM L-threonine. The AEC plates were incubated at 30 °C for 4 to 7 days.

4.3.5 Screening for high lysine producers amongst mutants obtained from B.lactofermentum ATCC 13869, BL 1136 and B57

The method described in section 3.3.3 was followed except that each mutant tested for lysine production was grown for 48 h on a medium I agar slant. A loopful of bacteria was then used to inoculate 30 mℓ of

seed medium. Some of the mutants obtained from B57 were screened using a modified screening medium (TNI medium) Appendix B.

4.3.6 Screening for high lysine producers among mutants obtained from EMS mutagenic treatment and by utilizing the L-lysine analogues LH, NEM and AEC

All mutants were screened as described in section 4.3.5 except that the screening medium used was TNIY medium. This is similar to the TNI medium used previously except that it obtained 1% yeast extract instead of 1% NZ-amine as the amino acid source.

4.3.7 Methods of lysine determination

The paper chromatographic method of Walczak and Oberman (1980) was utilized. Where high lysine yields were obtained from various mutants, these results were verified using the Technicon TSM amino acid analyser. (Ertingshausen and Adler, 1970.)

4.3.8 Effects of differing concentrations of AEC on the growth of D14, BL 1136, B57 and B.lactofermentum ATCC 13869

Loopfuls of bacteria from 48 h medium I agar slants of D14, BL 1136, B57 and the wild type parent strain ATCC 13869 were used to inoculate 30 mℓ lots of medium I in 250 mℓ Erlenmeyer flasks. The inoculated seed media were incubated at 30 °C on an orbital shaker at 170 rpm for 24 h. The seed media (0,1 mℓ) were then used to inoculate 30 mℓ aliquots of medium 2 supplemented with 4 mM L-threonine and concentrations of AEC of 8 mM, 16 mM, 24 mM and 32 mM respectively. Controls, with no AEC and threonine as well as uninoculated blanks were included. The inoculated flasks were incubated at 30 °C on an orbital shaker for 24 h. The absorbances were then recorded at 562 nm on a Unicam UV spectrophotometer.

4.3.9 Enzyme studies of B.lactofermentum ATCC 13869, BL 1136, B57 and D14

The aspartate kinase activities of the mutant strains as well as of the wild type parent strain were examined.

4.3.9.1 Preparation of a yeast extract

The aspartate kinase assay of Black (1962) is well documented with respect to a crude yeast extract. Thus in order to test the effectivity of the assay it was decided to first test it on a yeast extract.

Yeast cake (100 g) was crumbled and frozen by dropping into liquid nitrogen contained in a 250 mℓ beaker. The frozen yeast was stored at - 20 °C until used.

In order to make an extract, 88 mℓ of distilled water was added to the frozen yeast and the mixture warmed to 0 °C. Concentrated NH_4OH was added (0,88 mℓ) and the mixture stirred slowly at 4 °C for 15 h and centrifuged at 10 000 rpm for 15 min. to yield 115 mℓ of extract.

4.3.9.2 Preparation of bacterial extracts

Cells were prepared according to the method of Shiio and Ujigawa (1978). A loopful of the wild type strain or one of the mutants from 48 h medium I slants was used to inoculate 50 mℓ of TNIY medium in 500 mℓ Erlenmeyer flasks. Four such flasks were inoculated with each organism. They were left on an orbital shaker at 30 °C for 24 h (until the optical density at 562 nm reached approximately 10,0). The cells were then harvested by centrifugation and washed twice with 0,2% ($^{m}/v$) KCI and resuspended in 5,0 mℓ of 0,05 M Tris.HCI buffer pH 7,5. The resuspended cells were then pooled to give a total volume of 20 mℓ.

4.3.10 Methods of cell disruption

In order to determine the best technique for obtaining crude extracts from B.lactofermentum, the wild type strain ATCC 13869 was exposed to various methods of cell disruption. Plate counts were done before and after each treatment to determine percentage viability. A protein assay was also carried out on each extract according to the method of Lowry et al (1951), and extracts were also assayed for enzyme activity using a marker enzyme from the Krebs cycle. (Section 4.3.11).

4.3.10.1 French pressure cell

A 20 mℓ aliquot prepared as described in section 4.3.9.2 was subjected to 89 kN pressure per 2,5 cm^2 in a French pressure cell model J4-3398A using an Amsler press. The cell suspension was collected, kept on ice and centrifuged at 10 000 rpm for 30 min. The supernatant was used as a crude enzyme preparation.

4.3.10.2 Sonication

A 20 mℓ aliquot prepared as described in section 4.3.9.2 was divided into two 10 mℓ aliquots. One was subjected to sonication for 3 min. at 120 watts on a Branson sonicator with a standard 1,27 cm disrupter horn. The other aliquot was sonicated for 20 min at 120 watts. Both samples were cooled during sonication by an ice/salt bath. The two samples were then centrifuged at 10 000 rpm for 30 min. and the supernatants stored at -5 °C until used as crude enzyme extracts.

4.3.10.3 Sonication using a protective agent

Two 10 mℓ aliquots were obtained as described in Section 4.3.9.2. To one aliquot 30% (^{v}/v) glycerol was added and to the other 1% (^{m}/v) Bovine Serum Albumin. The samples were sonicated at 120 watts for 15 min. and cooled in an ice/salt bath. Foaming was found to be a problem if longer sonication periods were attempted. The samples were centrifuged and stored at - 5°C.

4.3.10.4 Lysozyme treatment in addition to sonication

Two 10 mℓ aliquots were obtained (Section 4.3.9.2). To each sample, 100 µg/mℓ lysozyme was added and the suspension was stirred on ice for 20 min. One aliquot was sonicated for 4 min. and the other aliquot for 15 min. Both samples were cooled in an ice/salt bath during sonication. Samples were centrifuged and stored at - 5 °C.

4.3.10.5 Braun cell disintegrator

A 20 mℓ aliquot obtained as described in section 4.3.9.2 was placed in an 80 mℓ aluminium container together with 50 g of acid washed glass beads of 150 to 200 microns in size (Merkenschlager et al 1957). The cells were disrupted in a Braun cell disintegrator at maximum speed for 2x one minute intervals. Carbon dioxide was passed around the container during disruption to prevent the temperature exceeding 5 °C. The samples were centrifuged and the supernatants stored at - 5 °C.

A 20 mℓ aliquot of yeast extract obtained as described in section 4.3.9.1 was disrupted in a similar manner, except that 50 g of glass beads of 450 to 500 microns in size were used (Merkenschlager et al 1957) and the cells were disrupted at maximum speed for a total of 60 seconds. The samples were centrifuged and the supernatant used as a crude enzyme extract.

4.3.11 Enzyme assay proceedures

4.3.11.1 Malate Dehydrogenase (E.C. 11.1.37)

Each extract obtained from the various methods of cell disruption was assayed for malate dehydrogenase (MDH) activity according to the method of Boehringer Mannheim (1973). Malate dehydrogenase was chosen as a marker enzyme as it is a major enzyme in the Krebs cycle, feeding oxaloacetate into lysine synthesis and is easily assayed without

prior purification of the crude extract. Malate dehydrogenase specific activities were also recorded in the mutant strains for comparative purposes.

Malate dehydrogenase was assayed by the oxidation of NADH (0,2 mM) in the presence of oxaloacetate solution (0,5 mM) and cell free extract. The buffer used was phosphate pH 7,5 (94 mM) and the final volume in the cuvette was 3,0 mℓ. The reactions occurring in the cuvette are illustrated as follows:

$$1/\text{oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-malate} + NAD^+$$

The change in absorbance at 340 nm per unit time is a measure of malate dehydrogenase activity. This was expressed as nmoles/min/mg protein. The assay was carried out at 25 °C in 1 cm cuvettes. Absorbance was measured using a Pye Unicam SP 6-550 UV spectrophotometer and recorded every 30 seconds.

4.3.11.2 Aspartate kinase (E.C. 2.7.2.4)

Aspartate kinase (AK) activity was measured according to the methods of Tosaka and Takinami (1978) and Truffa-Bachi and Cohen (1970). The assay mixture contained the following components in a total volume of 2,0 mℓ:

L-aspartate (50 µm), ATP (30 µm), $MgSO_4.7H_2O$ (20 µm), hydroxylamine (500 µm), ammonium sulfate (400 µm), Tris H_2SO_4 buffer pH 7,5 (100 µm) plus cell free extract. After incubation at 37 °C for 1 h the reaction was stopped by addition of 3,0 mℓ $FeCl_3$ reagent, (10% $FeCl_3.6H_2O$, 3,3% trichloracetic acid and 0,7 N HCl) (Black, 1962).

After centrifugation the absorbance was measured at 540 nm. A blank reaction mixture without L-aspartate served as a control. The aspartokinase activity was determined by measuring the amount of aspartohydroxamate formed in 1 hour. This was expressed as n moles/min/mg protein. The molar extinction of β-aspartohydroxamic acid under these conditions is 600 (Black, 1962).

4.3.11.3 Optimization of aspartate kinase activity

It was decided to use the Braun cell disintegrator to obtain crude enzyme extracts. Aspartate kinase activity was detected from crude yeast extracts in this manner. However, when the crude bacterial extracts were assayed very little aspartate kinase activity could be detected after 1 hour. If the assay was left for 5 hours then measurable amounts of aspartate kinase could be obtained. It was therefore decided to do an ammonium sulfate precipitation on the extracts. The cell free extracts were mixed at 0 °C with 5 volumes of saturated ammonium sulfate solution. After stirring for 30 min. at 0 °C the precipitates were collected by centrifugation at 10 000 rpm for 15 min. and dissolved in the same buffer. This solution (approximately 15 to 20 mg/mℓ protein) obtained from B.lactofermentum ATCC 13869 and the various mutants was used as the crude enzyme preparation for the aspartate kinase assay.

Stadtman et al (1961) found the relationship between enzyme concentration and the amount of aspartohydroxamate produced to be linear over only a very narrow range, and that it deviated sharply with increasing enzyme concentrations above a certain level.

In a similar test of linearity, deviations occurred at very low enzyme concentrations. Furthermore, enzyme activity fell off markedly after prolonged incubation. It was therefore decided to standardize the assay conditions, so that the quantity of enzyme used produced an absorbance in the range of 0,2 - 0,6 after an incubation period of one hour.

4.3.11.4 In vitro aspartate kinase inhibition studies

The properties of the aspartate kinase of B.lactofermentum ATCC 13869 as well as the mutants BL 1136, B57 and D14 were examined in vitro. The assay methods were the same as described in section 4.3.11.2 except that 1 mM, 5 mM and 10 mM concentrations respectively of L-lysine and L-threonine were added to the enzyme reaction mixtures as well as 1 mM and 10 mM concentrations of threonine plus lysine in a total reaction volume of 2,0 mℓ.

4.4 RESULTS

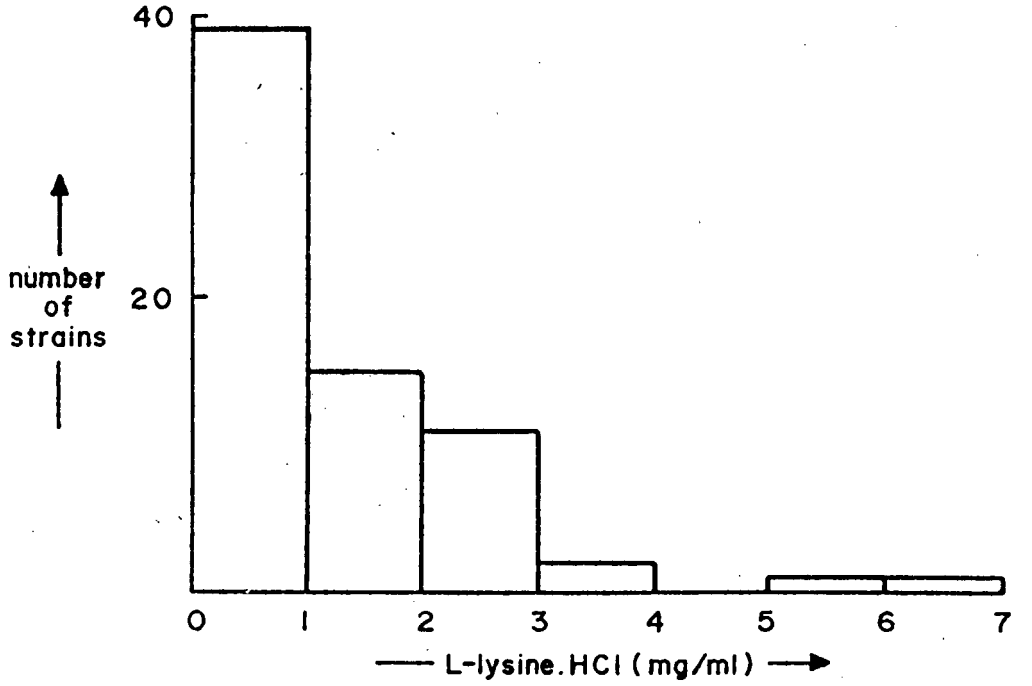
4.4.1 Analogue resistant mutants obtained from B.lactofermentum ATCC 13869, BL 1136 and B57

A total of 69 mutants were isolated from ATCC 13869 and screened for potentially high lysine producers (fig. 11). The wild type parent strain gave 0,2 mg/mℓ L-lysine.HCl on the screening medium while one of the best mutants obtained, A4089 gave 6,5 mg/mℓ L-lysine.HCl on the same medium. However when this mutant was retested on the screening medium it appeared to have partially reverted and gave only 2,5 mg/mℓ L-lysine.HCl.

A total of 190 mutants from BL 1136 were screened for potential high lysine producers (fig. 12). The hse$^-$ parent strain gave 9,7 mg/mℓ L-lysine.HCl on the screening medium while one of the best mutants obtained, A21 gave 16,3 mg/mℓ L-lysine.HCl on the same medium. However when this mutant was later retested on the screening medium it only gave 6,4 mg/mℓ L-lysine.HCl.

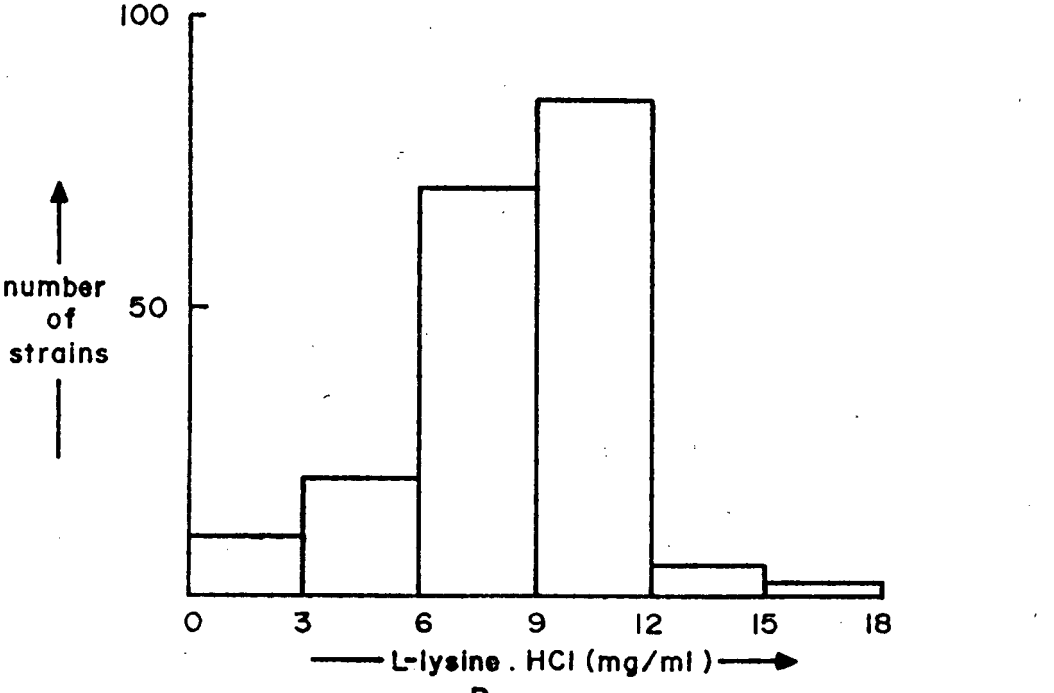
A total of 236 mutants were isolated from B57 and screened for high lysine production (fig. 13 to 16). The hse$^-$leu$^-$ parent strain gave 14,9 mg/mℓ L-lysine.HCl on the screening medium. One of the best mutants obtained, BA 173 gave 19,0 mg/mℓ L-lysine.HCl on the same medium. This was an increase of approximately 21%. However when this mutant was later retested on the screening medium it gave only 6,6 mg/mℓ L-lysine.HCl. This mutant was obtained when it was exposed to 200 µg/mℓ NTG for 20 min. and plated onto medium 2 agar plates supplemented with 4 mM AEC and threonine respectively.

It was postulated that the basic screening medium of Nakayama et al (1961) used for all the previous mutants might not be suitable for detecting AEC resistant strains which were high lysine producers. It was thought that as this medium contained relatively low glucose levels this might result in low lysine yields, which in turn might not immediately illustrate the benefits of the AEC resistant genotype among



Distribution of AEC^R mutants derived from wild type strain ATCC 13869 with respect to the production of L-lysine.

FIG. 11



Distribution of AEC^R mutants derived from strain BL 1136 with respect to the production of L-lysine.

FIG.12

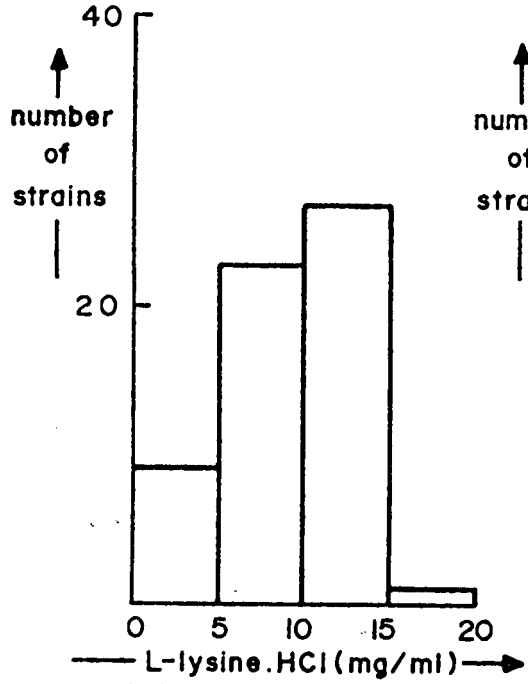
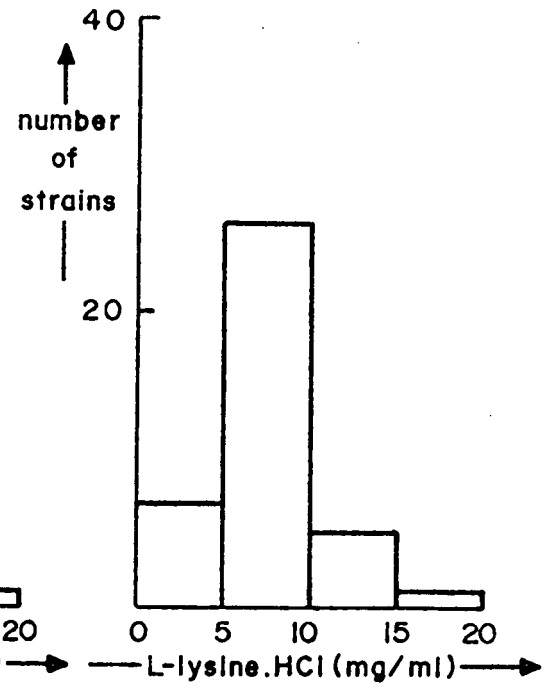


FIG.13 (i) 200 μg/ml NTG used
(ii) 2,5 mg/ml AEC + 2,5 mg/ml L-Threonine



(i) 200 μg/ml NTG used
(ii) 4mM AEC + 4 mM L-Threonine FIG.14

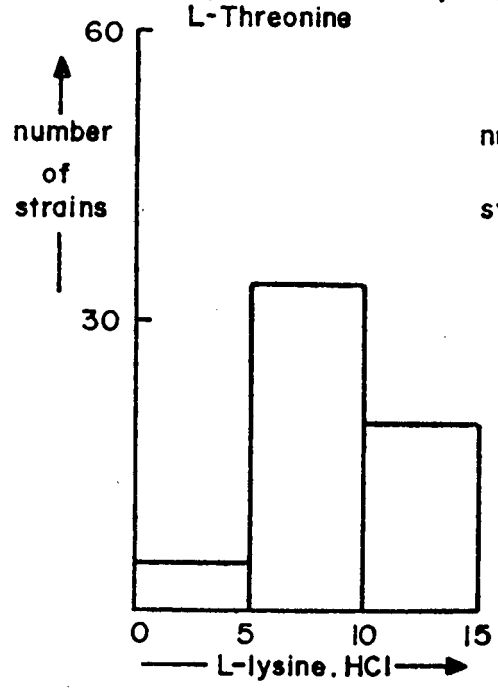
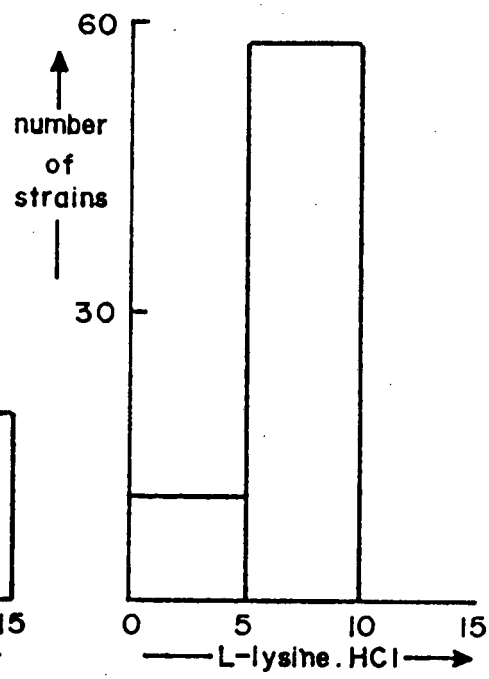


FIG.15 (i) 250 μg/ml NTG used
(ii) 4 mM AEC + 4 mM L-Threonine.



(i) 250 μg/ml NTG used
(ii) 2,5 mg/ml AEC + 2,5 mg/ml L-Threonine. FIG.16

Distribution of AEC^R mutants derived from B57 with respect to the production of L-lysine.

the mutants screened. It was also thought that the growth of the mutants could be limited due to insufficient nutrients in the medium. A total amino acid analysis was performed on NZ-amine, the sole amino acid source in the screening medium (Appendix C). The levels of the amino acids essential to the auxotrophs such as methionine, leucine and threonine were according to the literature (Nakayama, 1972) possibly too limiting to support sufficient growth.

A new screening medium was devised, TNI medium. It differed from the original screening medium in that it contained 10% (^{m}/v) glucose as opposed to 7,5% (^{m}/v), as well as 1% (^{m}/v) NZ-amine in place of 0,5% (^{m}/v). Trace elements of Fe^{2+} and Mn^{2+} as well as thiamine were also added. A further 60 mutants were isolated from B57 (fig. 17) and screened on this medium together with B57 and BL 1136 (Table 5). One of the best mutants obtained was D14 which gave 18,5 mg/mℓ L-lysine.HCl on the TNI screening medium.

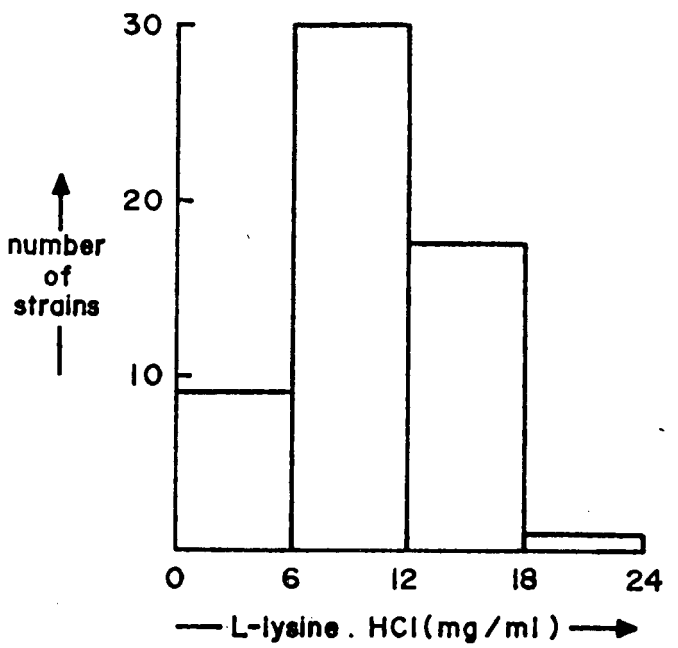
As no reliable results were obtained with the lysine determination methods of Chinard (1952) and Gaillardin et al (1975), the mutants were assayed by decending paper chromatography and the most promising results verified using the Technicon TSM amino acid analyser (Ertingshausen and Adler, 1970).

4.4.2 Mutants isolated from B57 using various lysine analogues

A total of 18 NEM^{R} mutants and 14 LH^{R} mutants were obtained from B 57 and screened together with 8 AEC^{R} mutants on TNIY screening medium (fig. 18 - 20).

B57, BL 1136 as well as D14 were grown on this medium for comparative purposes (table 6). The 5 most promising mutants obtained were found to give slightly in excess of 18 mg/mℓ L-lysine.HCl. Although these mutants were superior to the parent strain B57, none of them were significantly better than D14.

Roughly the same number of mutants were screened from LH and NEM supplemented plates. When compared it was found that a large number



Distribution of AEC^R mutants derived from strain B57 with respect to the production of L-lysine.

FIG.17

Strain	Genotype	L-lysine.HCl (mg/mℓ)
BL 1136	hse^-	16,6
B 57	hse^-leu^-	15,2
D 14	hse^-leu^- AEC^R	18,5

Table 5

Mutants BL 1136, B57 and D14, the best AEC^R mutant obtained screened on TNI medium

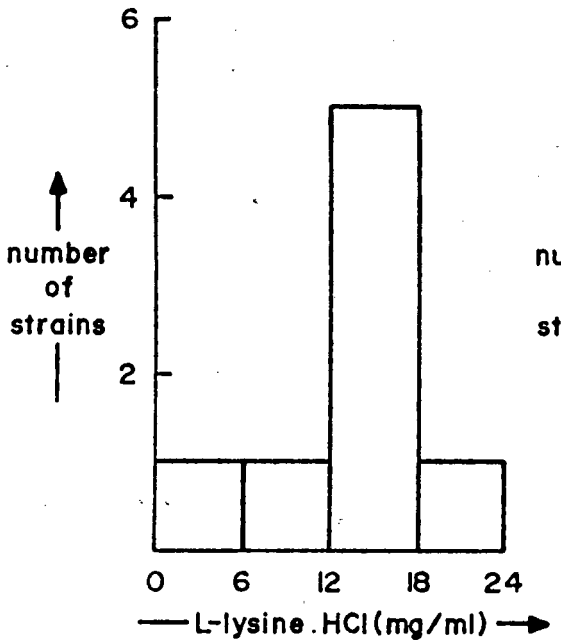
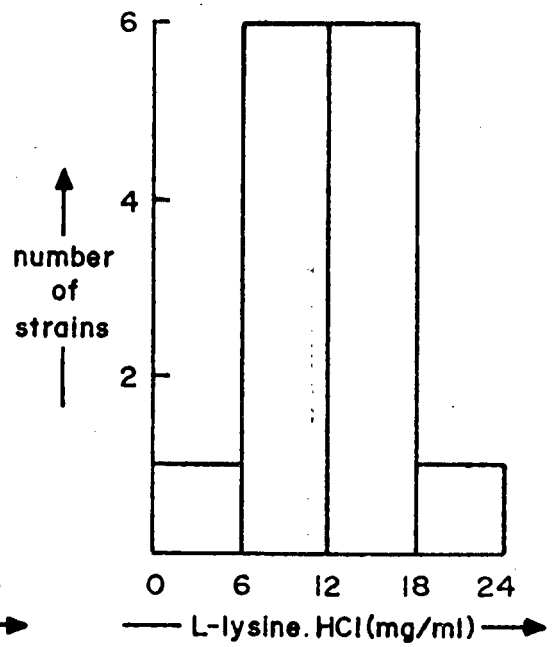


FIG.18 AEC^R strains



LH^R strains FIG. 19

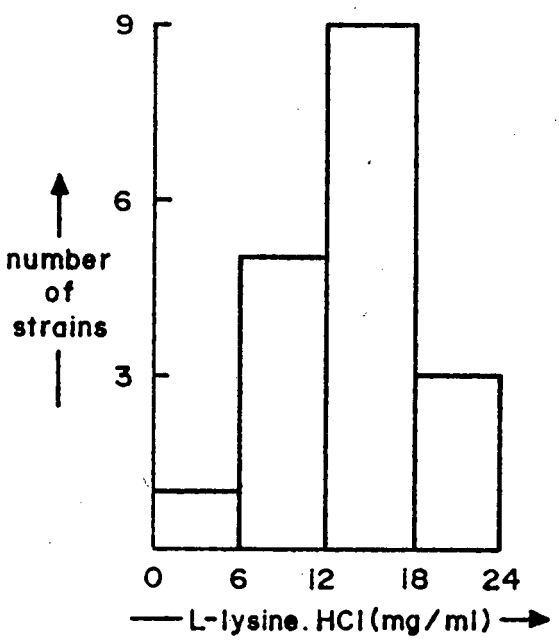


FIG.20 NEM^R strains

Distribution of analogue^R strains derived from B57 with respect to the production of lysine.

Strain	Genotype	L-lysine.HCl (mg/mℓ)
D 14	hse^-leu^- AEC^R	19,2
B 57	hse^-leu^-	16,3
BL 1136	hse^-	17,2

Table 6

Mutants D14, B57 and BL 1136 screened on TNIY medium

of higher lysine producing mutants were obtained from agar plates supplemented with NEM as opposed to LH. Fewer mutants obtained from AEC supplemented plates were screened and thus these results could not be directly correlated with results obtained from mutants selected on LH and NEM supplemented plates.

4.4.3 Analogue resistant mutants isolated from D14 after EMS treatment

It was thought possible to enforce the AEC^R genotype of D14 and thereby increase lysine yields, by exposing it to a different mutagen and re-selecting AEC^R mutants (fig. 21). A total of 18 mutants were obtained from D14 and screened on TNIY medium. Five of the most promising mutants obtained gave slightly more than 18 mg/mℓ L-lysine.HCl, but none, however showed any significant improvement over the parent strain D14.

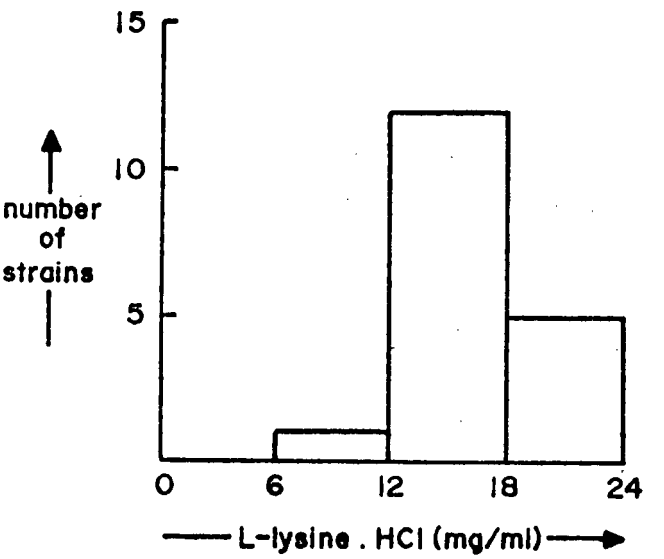
4.4.4 The effects of differing concentrations of AEC on the growth rates of B57, BL 1136, D14 and B.lactofermentum ATCC 13869

When D14, B57, BL 1136 and the wild type parent strain were grown in a medium to which increasing concentrations of AEC were added, the growth of D14 was scarcely affected while the growth rates of the other mutants and the wild type was decreased by between 70% and 90% (fig 22).

4.4.5 Comparison of various methods of cell disintegration of B.lactofermentum ATCC 13869

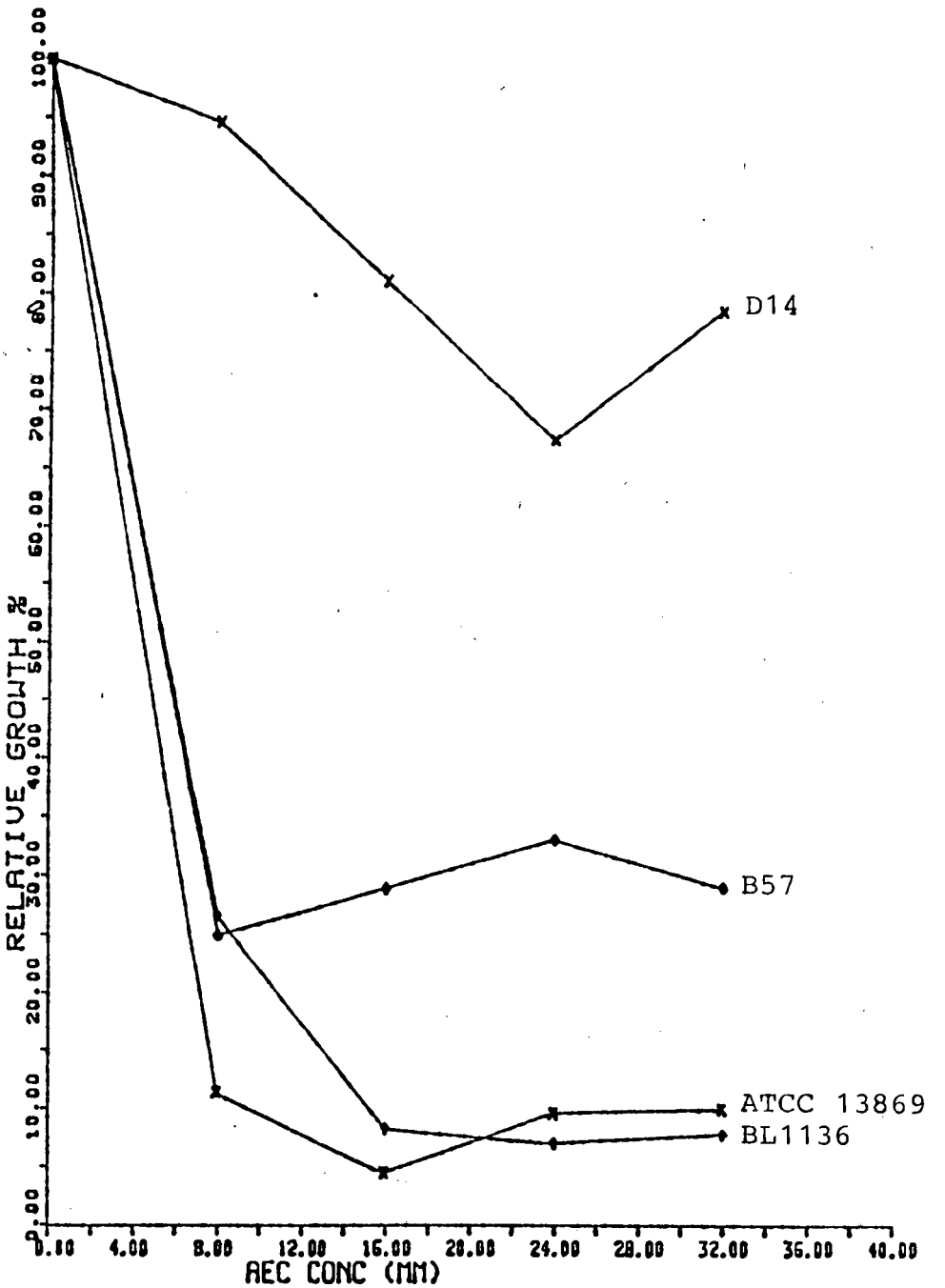
The different cell disintegration methods were compared regarding percentage viability of the cells, protein concentration in the extract and enzyme activities (Table 7).

It was decided to use the Braun cell disintegrator for obtaining crude extracts in further studies as it decreased viability by almost 100% and the protein and specific activity of the extract proved relatively high.



Distribution of AEC^R mutants derived from strain D14 with respect to the production of L-lysine.

FIG. 21



Effect of AEC on the growth of B. lactofermentum ATCC 13869, BL1136, B57 and D14

FIG. 22

Methods of cell disintegration	% Vialibity	Protein (mg/mℓ)	MDH Activity (U/mℓ)	MDH Specific Activity (U/mg)
French Pressure cell	7,9%	4,9	10,8	2,2
Sonication (3 min.)	6,8%	6,7	5,2	0,8
Sonication (20 min.)	0,4%	11,4	13,6	1,2
Sonication (15 min.) + glycerol	0,4%	11,9	13,1	1,1
Sonication (15 min.) + Bovine Serum Albumen	0,2%	11,9	15,5	1,3
Sonication (4 min.) + Lysozyme (100 ug/mℓ)	5,1%	8,0	13,3	1,7
Sonication (15 min.) + Lysozyne (100 ug/mℓ)	8,1%	4,9	8,0	1,6
Braun Cell Disintegrator	0,3%	10,2	18,0	1,8

Table 7
Comparison of various methods of cell disintegration of B.lactofermentum ATCC 13869

4.4.6. Specific activities of aspartate kinase and malate dehydrogenase in lysine producing mutants

Malate dehydrogenase specific activity was found to be highest in the wild type strain, while the specific activities of the mutants BL 1136, B57 and D14 proved to be fairly uniform (table 8).

In contrast the aspartate kinase specific activities varied widely between the wild type and the mutants. D14 had the highest specific activity followed by B57 and BL 1136, respectively the next highest lysine producers. The aspartate kinase specific activity of D14 was increased by 240% over the wild type and approximately 60% over the parent strain B57.

4.4.7 In vitro aspartate kinase inhibition studies

The effect of various concentrations of L-lysine and L-threonine on the aspartate kinase activities of B.lactofermentum ATCC 13869, BL 1136, B57 and D14 were studied (table 9).

The relative aspartate kinase activities of all the mutants including the wild type were affected in much the same way with the additions of L-lysine and L-threonine. The activities decreased with increasing L-lysine plus L-threonine concentrations.

Tosaka and Takinami (1978) claimed that 1 mM L-threonine inhibited the aspartate kinase activity of B.lactofermentum by 41%. In this case however, L-threonine (1 mM to 10 mM) was found to stimulate the aspartokinase activity of B.lactofermentum ATCC 13869 and its high lysine producing mutants, by more than 100% in certain cases.

Strain number	Property for selection	Enzyme Activity (nmoles/min/mg protein)	
		MDH *	AK **
ATCC 13869	wild type	11210	6,1
BL 1136	hse^-	4170	8,0
B 57	$hse^- leu^-$	4890	13,1
D 14	$hse^- leu^-$ AEC^R	4980	20,8
Yeast		2910	4,4

* MDH = malate dehydrogenase

** AK = aspartate kinase

Specific enzyme activities of yeast and selected lysine producing B.lactofermentum mutants

Table 8

Amino acids Added	Relative Aspartate kinase activities (%)			
	ATCC 13869	BL 1136	B 57	D 14
Control	100	100	100	100
1 mM lysine	85	92	91	95
5 mM lysine	62	84	76	81
10 mM lysine	59	67	69	68
1 mM lysine + 1 mM threonine	32	57	47	36
10 mM lysine + 10 mM threonine	19	45	22	11
1 mM threonine	132	202	180	159
5 mM threonine	116	241	205	165
10 mM threonine	108	228	173	156

Effect of lysine and threonine on the aspartate kinase activity of various high lysine producing mutants

Table 9

4.5 DISCUSSION

An attempt was made to isolate analogue resistant mutants from the homoserine auxotroph BL 1136, the homoserine leucine double auxotroph B57 as well as from B.lactofermentum ATCC 13869. Once analogue resistant mutants were obtained from the wild type, auxotrophic mutants could then be isolated from this strain.

The lysine analogue S-(2-aminoethyl)-L-cysteine (AEC) was utilized. Unfortunately AEC resistance appeared to be unstable as virtually all the mutants when later retested gave lower lysine yields. However, when one of the best analogue resistant mutants, D14, isolated from B 57 was retested there was no significant drop in lysine production. A modified screening medium was utilized, TNI medium, which was better able to illustrate the lysine producing potential of the various mutants. When B57 and BL 1136 were grown on this medium they produced substantially increased concentrations of lysine. BL 1136 in particular showed great potential for optimization.

D14 was exposed to EMS and mutants re-selected on AEC supplemented agar plates. A few promising mutants were obtained but none showed any significant improvement over the parent strain. Possibly a greater degree of success could have been obtained if D14 had been exposed to a lower concentration of EMS for a longer time period. Plachy (1968), Honzova et al (1968), Necásek and Plachy (1970) found this to be a more successful method when trying to obtain auxotrophic mutants.

None of the LH and NEM resistant mutants obtained from B57 were significantly better than D14 although some of the mutants did give slightly higher lysine yields than the parent strain B57.

When screening these mutants TNI medium was further modified to include yeast extract in place of NZ-amine. This was used for growing BL 1136, B57 and D14 for comparative purposes. They all produced increased lysine yields. In particular the lysine production by BL 1136 was suprising in view of its genotype, thus illustrating the latent ability of this bacterium to respond well to optimization.

D14 hse⁻leu⁻ AEC^R produced approximately 20% more lysine than its parent strain. This implies a substantial genetic improvement over B57 hse⁻leu⁻. In order to investigate the nature of this, the aspartate kinase of D14, the key enzyme in the regulation of the lysine biosynthetic pathway was investigated. When compared to that of the wild type strain, the specific activity of D14 aspartate kinase was 240% higher. This could account for its higher lysine producing potential. The AEC^R mutation of D14 enabling it to produce high lysine yields appears to be at the level of enzyme synthesis rather than the ability of the enzyme aspartate kinase to overcome repression by lysine and lysine plus threonine in the lysine biosynthetic pathway.

It was of interest to note that L-threonine alone stimulated the aspartate kinase activity of the wild type and the high lysine producing mutants isolated from it.

Miyajima and Shiio (1969) and Nakayama (1966) respectively reported that multivalent or concerted feedback inhibition occurred in Brevibacterium flavum and Corynebacterium glutamicum. Thus the aspartate kinase of these organisms was only inhibited when lysine and threonine were added simultaneously. However, Tosaka and Takinami (1978) reported that the aspartate kinase regulation of B.lactofermentum differed from that of B.flavum and C.glutamicum as it was inhibited by single or simultaneous additions of lysine and threonine.

The present studies however, indicate a different regulatory mechanism of the aspartate kinase of Brevibacterium lactofermentum ATCC 13869 and the high lysine producing mutants derived from it.

As D14 continued to remain relatively stable and produced acceptably high concentrations of lysine it was decided to continue using this mutant in further optimization and fermentation studies.

CHAPTER V

OPTIMIZATION STUDIES

5.1 SUMMARY

B57 hse⁻leu⁻ and D 14 hse⁻leu⁻ AEC^R were evaluated under controlled environmental conditions in 12 ℓ laboratory fermenters. They produced 12,7 mg/mℓ L-lysine.HCl after 183 h and 19,5 mg/mℓ L-lysine.HCl after 96 h respectively. During a fermentation run with D14 in a 20 ℓ fermenter incremental feeding was introduced and D14 was able to produce 32 mg/mℓ L-lysine.HCl after 73 h.

5.2 INTRODUCTION

During the screening programme described in earlier chapters, the chief concern was the rapid examination of a large number of cultures to determine their lysine producing capabilities on a comparative basis. Obviously the fermentation media chosen should be as nutritionally adequate as possible, providing the specific amino acids needed by the auxotrophic cultures. Too much attention cannot be directed towards optimization proceedures until a relatively small number of the potentially high yielding lysine producing cultures have been selected, since these cultures may well respond quite differently to attempts at optimization. This is apparent from the significant differences in the patent literature (Appendix A) with respect to fermentation media and operating conditions for the production of lysine by high yielding Corynebacterium and Brevibacterium strains.

Besides the importance of the nutritional requirements, control of aeration, agitation, temperature, pH and foaming can invariably lead to significantly higher fermentation efficiencies.

Whereas Erlenmeyer flasks are suitable for secondary screening and initial laboratory process development, they provide a poor estimate of the fermentation potential of a microorganism or its medium. This is chiefly due to relatively poor aeration. The importance of oxygen

supply to amino acid fermentations has been well documented. (Hirose et al 1978, Akashi et al 1979, Walczak and Oberman, 1980, Hilliger and Hänel, 1981). Furthermore pH control is difficult and the addition of $CaC0_3$ is less than satisfactory in maintaining a stable and controllable environment. The often desirable slow incremental feeding of certain nutrients such as glucose (Daoust, 1976) and protein hydrolysate (Beker et al, 1974) cannot be precisely controlled. For these reasons small mechanically stirred, air sparged, laboratory fermenters of 1 to 20 litres in size (plate 1) are preferred since more favourable aeration conditions are provided and both aeration and agitation can be varied to resemble more closely those of the larger production tanks.

Prior to obtaining the apparently stable AEC resistant mutant D14, the most promising auxotrophic mutant B57 was evaluated under controlled environmental conditions in a 12 ℓ laboratory fermenter.

Once the stability of D14 had been verified the performances of this mutant together with B57 and BL 1136 were compared. In an attempt to optimize the fermentation medium, the choice of amino acid source was carefully studied. Several potential sources of amino acids were analyzed regarding their amino acid compositions. Particular attention was paid to the amino acids threonine, methionine and leucine (Appendix C). Excess amounts of these amino acids together with high levels of biotin cause accumulation of lactate instead of lysine. Large amounts of homoserine or threonine in the presence of sufficiently small quantities of biotin result in glutamic acid production. Lysine is optimally produced at moderate biotin levels but with limited threonine (or homoserine). If both biotin and threonine (or homoserine) are limited, glutamic acid and lysine are accumulated together although in reduced yields (Nakayama, 1972). However, limiting the required amino acids too much could seriously affect the growth and the corresponding lysine production of the microorganism. As yeast extract proved to be the richest source of amino acids, it was decided to incorporate this into the fermentation medium, but to prevent a build up of threonine, methionine and leucine it was decided to feed the yeast extract incrementally into the fermentation medium.

Fermentation of D14 in a 20 ℓ laboratory fermenter, with incremental feeding of yeast extract

plate 1

This was initially performed in Erlenmeyer flasks and the most promising mutant D14 later re-evaluated under a more precisely controlled environment in a 20 litre laboratory fermenter.

5.3. METHODS

5.3.1 Media buffers and routine reagents

These are detailed in the appendix.

5.3.2 Fermentation of B57 in a 12 ℓ laboratory fermenter

(i) Media

The seed medium was similar to that used when screening auxotrophs for high lysine producers. The fermentation medium is detailed in the appendix.

(ii) Inoculum

A 10% inoculum of B57 was used. Two 48 h medium I slants of B57 were each flooded with 4,0 mℓ of sterile distilled water. Each slant was used to inoculate 400 mℓ of seed medium in a 1 ℓ Erlenmeyer flask. The flasks were left on an orbital shaker at 30 °C for 24 h before being used to inoculate the fermenter.

(iii) Running conditions

A Braun 12 ℓ fermenter with a 7 ℓ working volume was used. The temperature was kept constant at 30 °C and the pH controlled at pH 7,2 ± 0,1 with the automatic addition of 4 M NaOH. Foam control was by the addition of antifoam, in this case 10% silicone oil was used. The air flow rate was maintained at 0,5 v/v/min. Agitation speed was 400 rpm.

(iv) Sampling

Samples were taken at 8 and 16 h intervals. The pH was noted and growth determined by turbidimetric measurement of the suitably diluted culture at 562 nm using a Pye Unicam Sp 6-550 UV spectrophotometer. The samples were centrifuged at 10 000 rpm for 15 min. and the supernatants were assayed for lysine production according to the

method of Chinard (1952). Residual sugar was determined as total reducing sugar. Samples were also assayed by paper chromatography according to the method of Walczak and Oberman (1980). Two spots were detected. The Rf value of one corresponded to the L-lysine standard, the other was however unknown.

The sample was again assayed and a diaminopimelate (DAP) standard was included. In the lysine biosynthetic pathway, DAP is the last product formed before lysine. The Rf value of the unidentified spot corresponded to that of the DAP standard. Thus DAP as well as lysine was being produced. The concentration of DAP in the sample could be calculated in a similar manner to that used for lysine, i.e. by utilizing a standard curve.

5.3.3 Fermentation of D14 in a 12 ℓ laboratory fermenter

(i) Media

The seed medium was medium I and the fermentation medium was TNIY medium with 10% (^{m}/v) glucose and 1% (^{m}/v) yeast extract. The glucose was steralized separately before being added to the rest of the medium. The compositions of these media are detailed in the appendix.

(ii) Inoculum

A two stage inoculum was used. A 250 mℓ Erlenmeyer flask with 30 mℓ of medium I was inoculated with a loopful of D14 from a 48 h medium I slant.

After shaking for 24 h at 30 °C on an arbital shaker a 2%(^{v}/v) inoculum was used to inoculate 100 mℓ medium I in a 1 ℓ Erlenmeyer flask. This was again left at 30 °C on an orbital shaker for 24 h before inoculating the fermenter. Seven such flasks were needed to provide the 10% (^{v}/v) inoculum for the 7 ℓ working volume of the Braun fermenter.

(iii) Running conditions

These were the same as in section 5.3.2.

(iv) Sampling

The first sample was taken after 24 h and subsequent samples taken at approximately 8 and 16 h intervals. The pH was noted and the samples centrifuged at 10 000 rpm for 15 min. Dry mass determinations were done on the pellets in order to determine growth. The supernatants were assayed for lysine presence by the use of a Technicon TSM amino acid analyser. Residual sugar was determined as total reducing sugar.

The samples containing the highest concentrations of lysine were also assayed by paper chromatography in order to screen for the presence of DAP.

5.3.4 Incremental yeast extract and glucose feeding : Erlenmeyer flasks

(i) Inoculum

A loopful of cells from 48 h medium I slants of mutants BL 1136, B57, D14 and C.glutamicum ATCC 21513 were used to inoculate 30 mℓ aliquots of seed medium in 250 mℓ Erlenmeyer flasks. The inoculated flasks were left at 30 °C on an orbital shaker at 170 rpm for 24 h.A 10% inoculum was used to inoculate the fermentation medium.

(ii) Fermentation medium

Sixty millilitres of TNIY medium were dispensed into 500 mℓ Erlenmeyer flasks. The TNIY medium contained 0,5% yeast extract and 7,5% glucose. After 2 days 7,5% glucose plus 0,5% yeast extract was added to the medium. After 3 days a further 5% glucose was added to the fermentation medium. At each glucose addition a 5,0 mℓ sample was centrifuged at 10 000 rpm for 15 min. The supernatants were assayed

for L-lysine according to the method of Walczak and Oberman (1980) and later confirmed using the amino acid analyser. Residual sugar was determined as total reducing sugar.

5.3.5 Fermentation of D14 in a 20 ℓ laboratory fermenter with incremental feeding

(i) Media

The seed medium used was medium I. The fermentation medium was TNIY medium and initially contained 10% ($^{m}/v$) glucose and 0,5% ($^{m}/v$) yeast extract. During the run 1,58 ℓ of 50% ($^{m}/v$) glucose and 0,42 ℓ of 30% ($^{m}/v$) yeast extract solution was added incrementally. Thus the final concentration of sugar in 12 ℓ was 14,9% ($^{m}/v$) and the final concentration of yeast extract was 1,47% ($^{m}/v$).

(ii) Inoculum

A two stage inoculum was developed. A loopful of D14 from a 48 h medium I agar slant was inoculated into 30 mℓ of medium I in a 250 mℓ Erlenmeyer flask. The flask was left shaking at 30 °C on an orbital shaker at 170 rpm for 24 h. A 0,4 mℓ aliquot of this inoculum was placed in 100 mℓ of medium I in a 1,0 ℓ Erlenmeyer flask and left shaking on the orbital shaker for 24 h at 30 °C. Ten such flasks were pooled for the 10% ($^{v}/v$) inoculum. An extra flask was included in order to measure the pH and growth (A562 nm).

(iii) Running conditions

The fermentation was conducted in a Chemap 20 ℓ fermenter with a 12 ℓ working volume. The temperature was kept constant at 30 °C $\pm$ 1 °C and the pH at pH 7,2 with the automatic addition of 4 M NH_4OH. Foam control was by the addition of 10% ($^{v}/v$) silicone oil antifoam. Air flow rate and agitation speed were controlled so as to maintain an adequate dissolved oxygen content in the medium (viz. 10% saturation). The yeast extract plus half of the glucose solution was added between 20 h

and 44 h using a peristaltic pump with a flow rate of 0,29 mℓ/min. The rest of the glucose solution was incrementally fed into the fermenter between 44 h and 68 h in a similar manner. The flow rate was 1,1 mℓ/min.

(iv) Sampling

Samples were taken at intervals (see fig. 26). The pH was noted and the samples were centrifuged at 10 000 rpm for 15 min. Dry mass determinations were done on the pellets in order to determine growth and the supernatants were assayed for lysine production and presence of DAP according to the paper chromatography method of Walczak and Oberman (1980). These results were verified using the TSM Technicon amino acid analyser. A total amino acid analysis of the culture broth after 73 h was also performed using the analyser as set up for amino acid analysis in physiologic fluids (Ertingshausen and Adler, 1970). Residual sugar was determined as total reducing sugar.

5.4 RESULTS

5.4.1 B57 fermentation in a 12 ℓ laboratory fermenter

After 183 hours 12,7 mg/mℓ L-lysine.HCl was produced. However, only approximately half the sugar was utilized. A yield of 29% in respect of glucose consumed was achieved. (Fig. 23). Approximately 1,0 mg/mℓ of DAP was produced after 183 hours.

5.4.2 D14 fermentation in a 12 ℓ laboratory fermenter

After 96 hours 19,5 mg/mℓ L-lysine.HCl was produced and 4,1 mg/mℓ DAP. During the subsequant 24 hours lysine production began to plateau and after 120 hours 20,8 mg/mℓ L-lysine.HCl was produced (fig. 24) and approximately 4,0 mg/mℓ DAP. All the sugar was utilized giving a yield of 21%.

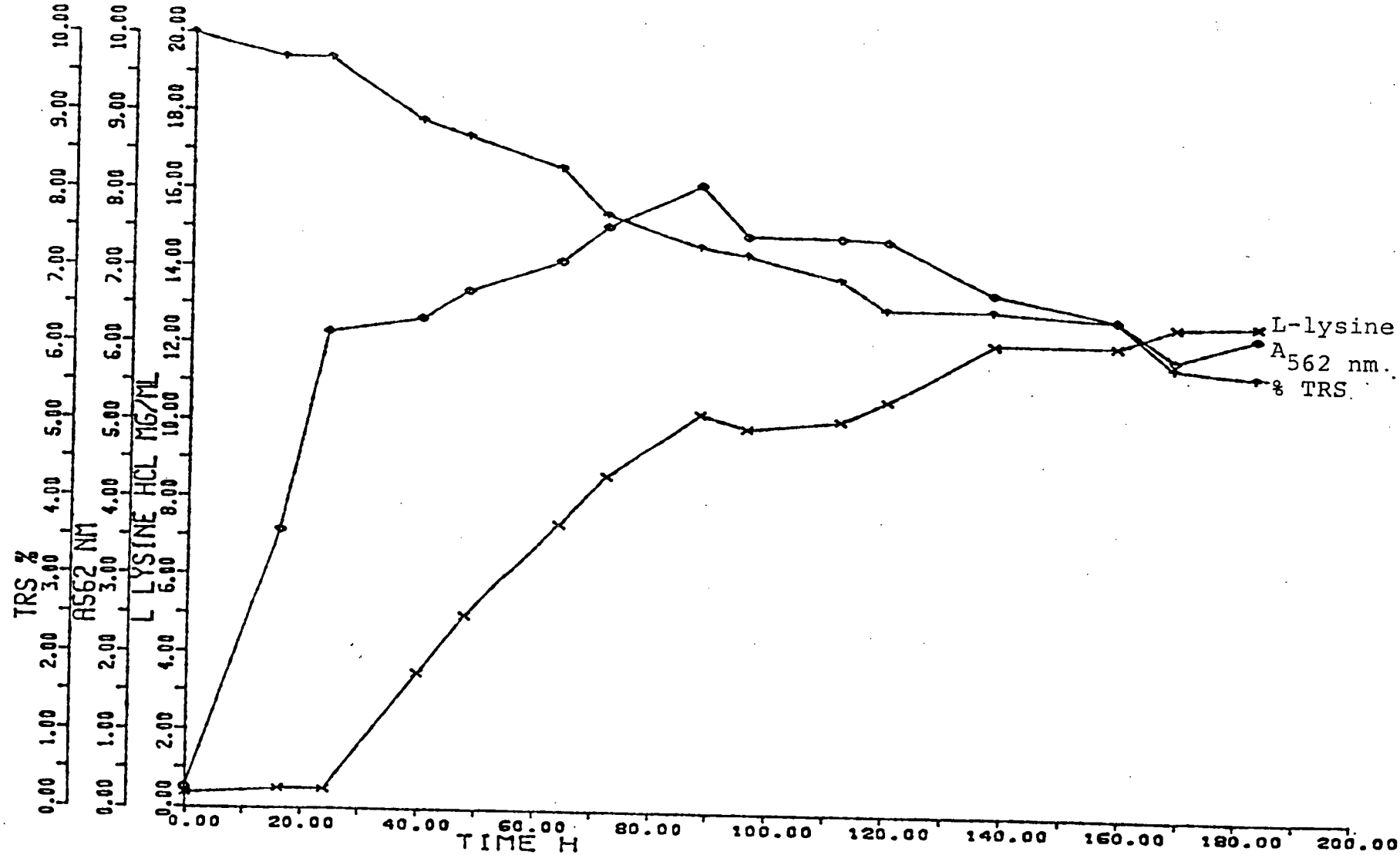
5.4.3 Incremental yeast extract and glucose feeding : Erlenmeyer flasks

D14 again proved to be a most promising mutant and produced 34,4 mg/mℓ L-lysine.HCl (fig. 25). The yields of both BL 1136 and B57 were also considerably improved over the results obtained on the standard TNIY medium (table 6). Although the mutant C.glutamicum ATCC 21513 was still considerably inferior to BL 1136 and D14 in respect of lysine production, the yield of 23,0 mg/mℓ obtained was suprisingly high in view of the poor results obtained with this mutant on the initial screening medium (section 3.4).

5.4.4 D14 fermentation in a 20 ℓ laboratory fermenter

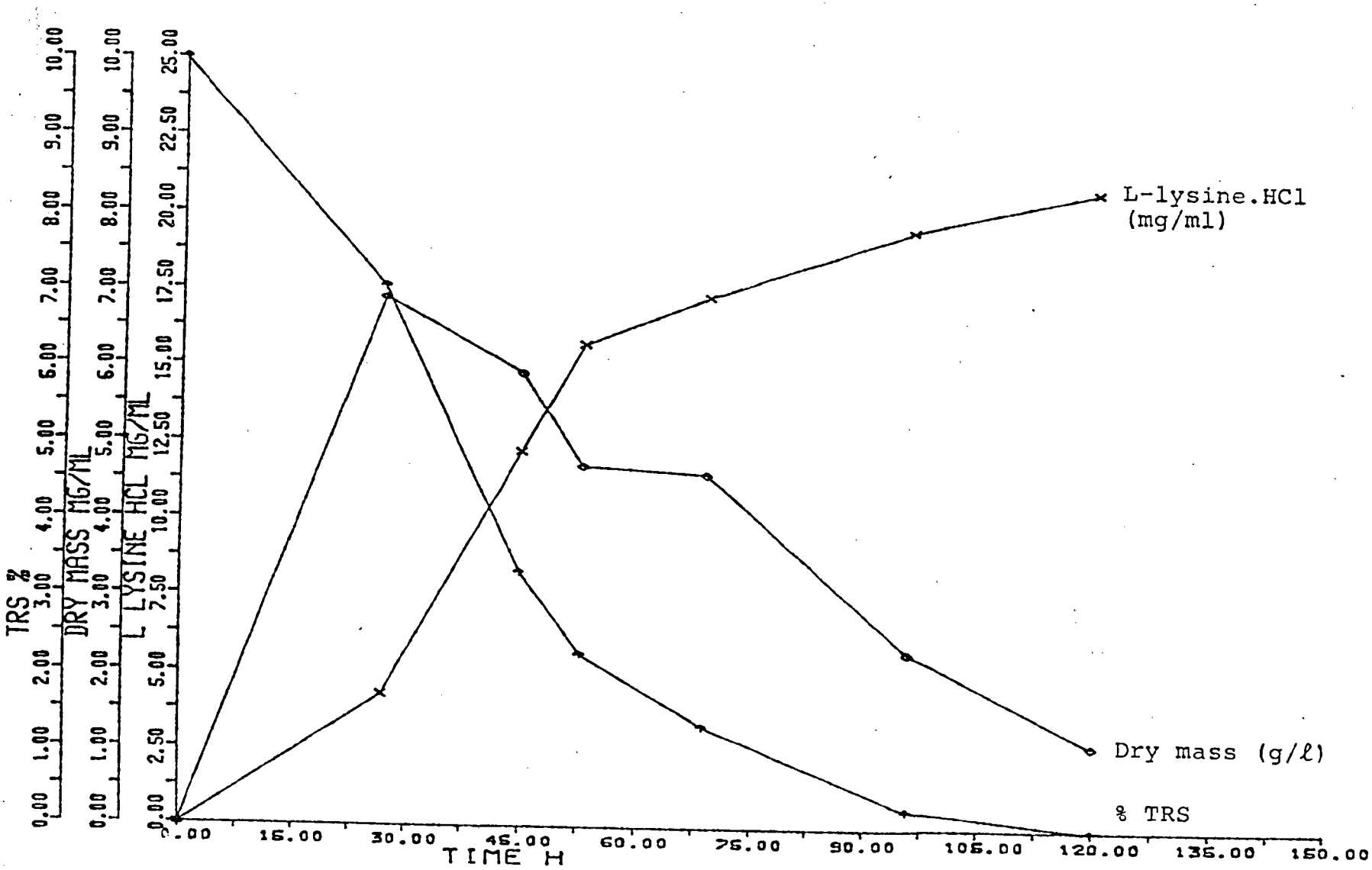
The dry mass of the two stage inoculum before it was added to the fermenter was 1,0 g/ℓ. After 73 hours almost all the sugar was utilized and 32 mg/mℓ L-lysine.HCl was produced (fig. 26). This was a yield of approximately 22%. No DAP could be detected.

A total amino acid analysis was carried out on a sample of the broth taken after 73 h (table 10). Lysine was produced virtually exclusively with only traces of other amino acids being formed.



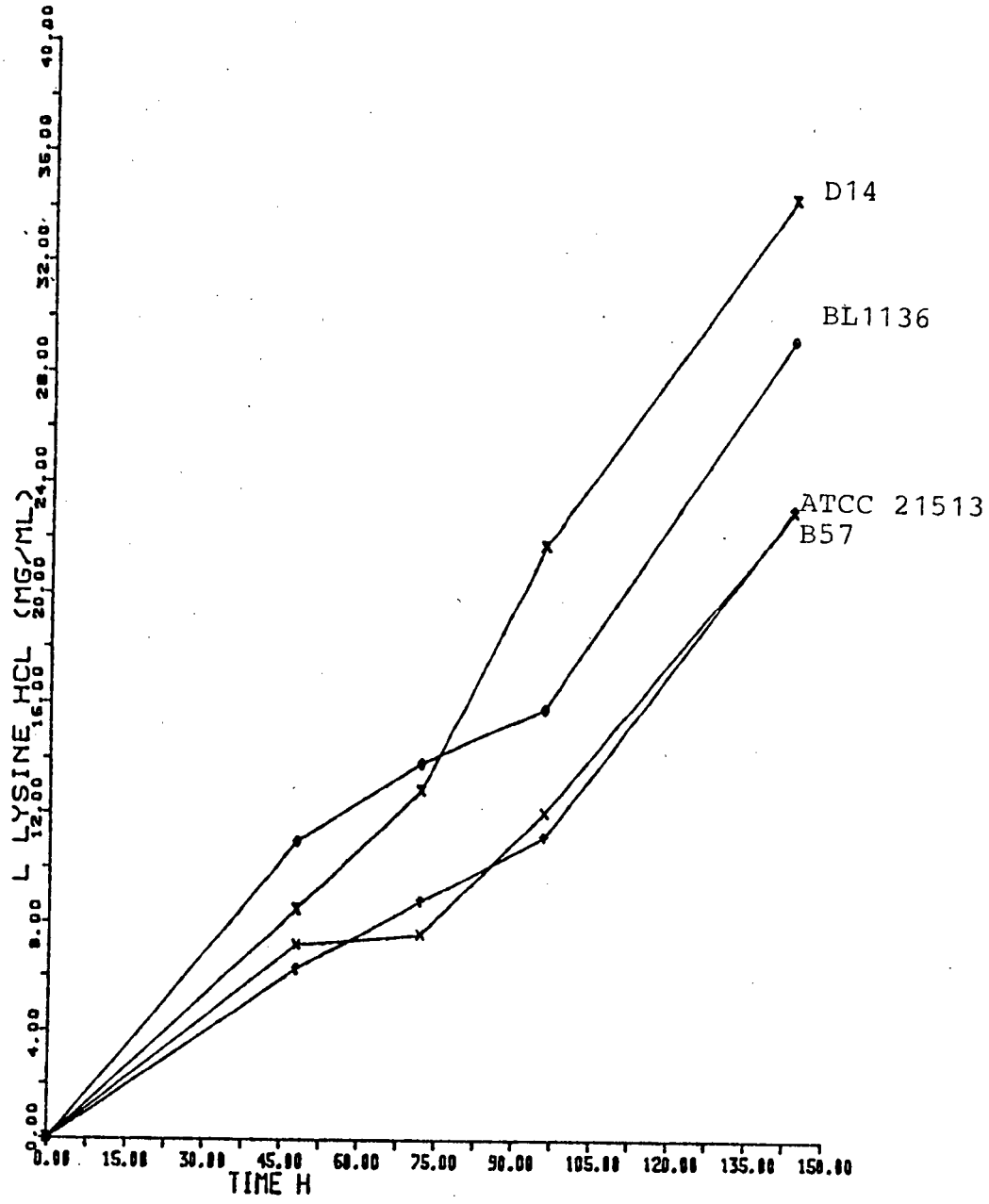
Fermentation profile of B57 in a 12ℓ laboratory fermenter

FIG. 23



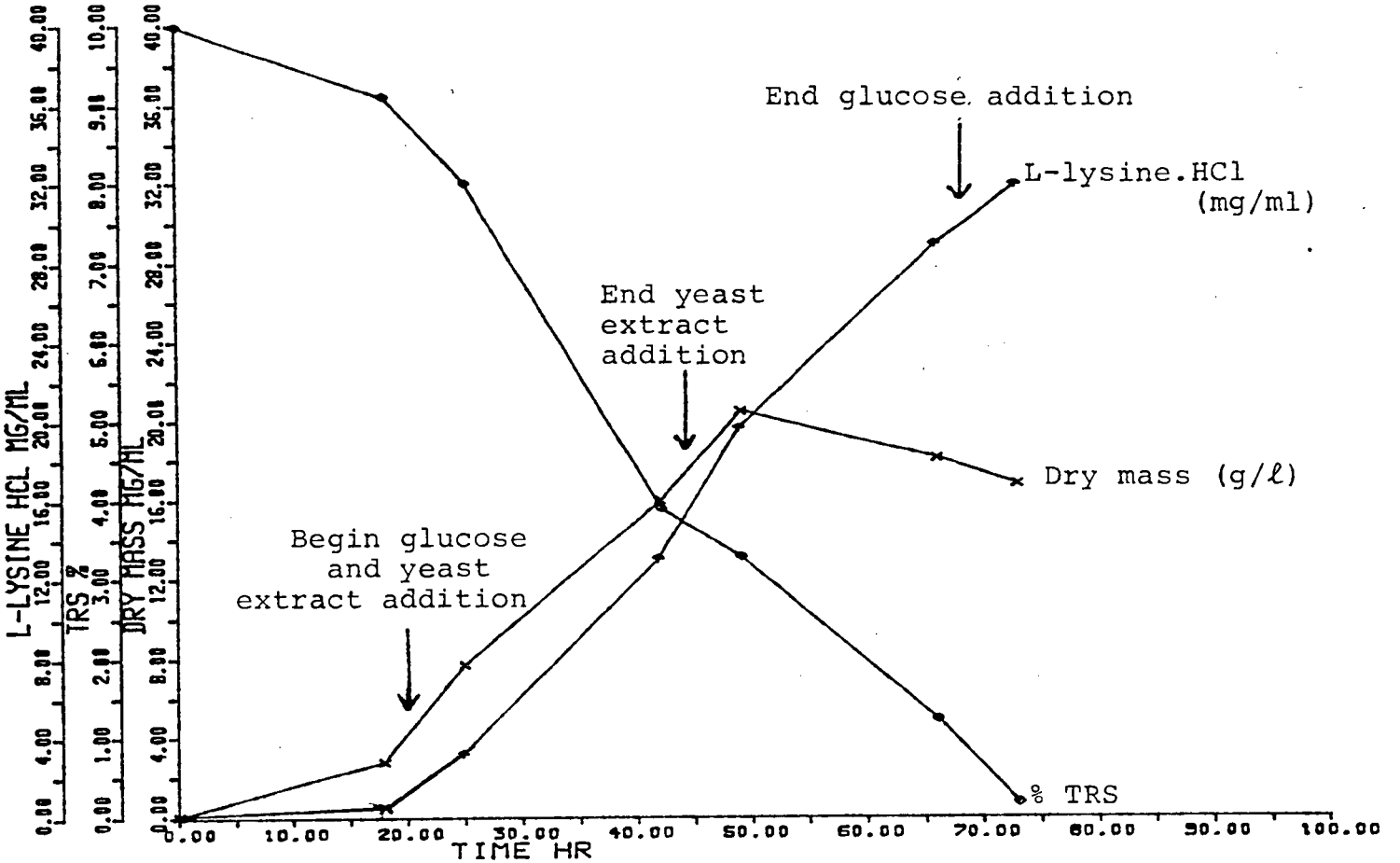
Fermentation profile of D14 in a 12ℓ laboratory fermenter

FIG.24



L-Lysine production by mutants subjected to incremental yeast extract and glucose feeding

FIG. 25



Fermentation profile of D14 in a 20ℓ laboratory fermenter

FIG.26

Amino Acid	Amino Acid mg/100 mℓ sample
Glutamic acid	19,7
Glycine	16,6
Alanine	47,3
Valine	124,5
Isoleucine	4,0
Leucine	7,7
Tyrosine	2,4
Phenylalanine	4,2
Lysine.HCl	3 200,0

Table 10

A total amino acid analysis of a broth sample taken after 73 h from a fermentation in a 20 ℓ laboratory fermenter utilizing D14

5.5 DISCUSSION

Yeast extract proved to be a particularly good amino acid nitrogen source for the production of lysine by selected mutants. In conjunction with incremental feeding of both the yeast extract and glucose the yields of C.glutamicum ATCC 21513, BL 1136 and D14 were all substantially increased in comparison with the results obtained on the standard screening medium in experiments also conducted in Erlenmeyer flasks. The highest yield obtained 34,4 mg/mℓ L-lysine.HCl for the homoserine leucine double auxotroph and AEC resistant mutant, D14 represents an 85 fold improvement in lysine production over the original wild type glutamate producing Brevibacterium lactofermentum (ATCC 13869).

The homoserine leucine double auxotroph C.glutamicum ATCC 21513 was also tested for comparative purposes. Although this mutant had previously performed very poorly on the standard screening medium giving 3,7 mg/mℓ L-lysine.HCI (chapter III) it did respond surprisingly well to optimization and a yield of 23,0 mg/mℓ L-lysine.HCl was obtained.

This is still, however, considerably inferior to the mutants BL 1136 and D14 obtained in this study. Nevertheless it does emphasize the individuality of mutants in their response to optimization and the severe limitation imposed through the use of a single medium during initial screening.

During trials conducted in laboratory fermenters the lysine levels obtained in Erlenmeyer flasks were maintained although no further increases were obtained.

When the AEC^R mutant D14 was tested in a 12 ℓ laboratory fermenter utilizing a richer fermentation medium and a two stage inoculum it was found to be able to produce 64% more lysine in a 64 hour shorter time period than its parent strain B57.

However the advantage of incremental feeding of yeast extract and glucose which was first illustrated utilizing Erlenmeyer flasks was further emphasized when applied to a laboratory fermentation of D14. Where no incremental feeding was carried out during a fermentation, 19,5 mg/mℓ L-lysine.HCl was produced by D14 after 96 h and 20,8 mg/mℓ L-lysine.HCl was produced after 120 hours. With incremental feeding, D14 produced 32 mg/mℓ L-lysine.HCl in only 73 h.

The time taken for the mutant D14 to reach maximum lysine production was significantly reduced from approximately 6 days (when Erlenmeyer flasks were used) to 3 days when a laboratory fermentation was conducted with incremental feeding.

It was of interest to note that during straightforward fermentations (i.e. without incremental feeding) measurable amounts of DAP were obtained together with the high concentrations of lysine. Thus under optimum lysine producing conditions DAP builds up and is the rate limiting step in the pathway. This could be due to low levels of DAP carboxylase present or actual inhibition of DAP carboxylase by high lysine levels. (Nakayama et al 1966).

However, during a fermentation where incremental feeding occurs there is no build up of DAP as the glucose is channelled into the lysine pathway at a slower rate. This could possibly account for the higher lysine levels obtained with incremental feeding.

CHAPTER VI

GENERAL DISCUSSION

In order to initiate a process for producing large quantities of lysine by fermentation, a suitable bacterium had to be found.

The regulatory mechanisms involved in the L-lysine biochemical pathway are less complex in the glutamate producing bacteria Brevibacterium and Corynebacterium than in Escherichia coli and consequently are more easily overcome by the appropriate mutations.

As described in Chapter I, the production of lysine is greatest by certain auxotrophic mutants, in particular homoserine leucine or homoserine alanine double auxotrophs. Lysine production is further increased when analogue resistant mutants are isolated from these auxotrophs.

Several homoserine, leucine and homoserine leucine double auxotrophs were obtained from the high glutamate producer B.lactofermentum ATCC 13869 and a member of the Corynebacterium genus C.glutamicum ATCC 13032. These mutants were screened for their ability to produce lysine.

A promising homoserine leucine double auxotroph B57 as well as a homoserine auxotroph BL 1136 were obtained from B.lactofermentum. The production of lysine by these mutants was increased approximately 40 fold over the wild type parent strain.

The wild type B.lactofermentum proved to be a good choice as a parent strain for obtaining high lysine producing auxotrophs. In a recent study conducted in South Africa (Perrow, 1982) many attempts were made to obtain high lysine producing mutants from B.flavum. These were relatively unsuccessful and the lysine concentrations obtained from these mutants could not be increased much beyond 8,0 mg/mℓ.

Analogue resistant mutants were isolated from B.lactofermentum and with the aid of a richer screening medium D14 was obtained which proved to be relatively stable and produced approximately 20% more lysine than its parent strain B57.

Further mutational work involving D14 and B57 utilizing the mutagen ethyl-methane-sulphonate in place of N-methyl-N'-nitro-N-nitrosoguanidine and different lysine analogues (L-lysine hydroxamate and N-ε-methyl-L-lysine.HCl) yielded a new set of mutants which gave good lysine yields but none significantly better than D14. These mutants could however be used in lieu of D14 should reversion become a problem in the future.

In order to elucidate the nature of the AEC^R mutation of D14, the aspartate kinase of this mutant as well as of BL 1136, B57 and the wild type B.lactofermentum ATCC 13869 was investigated.

The aspartate kinase specific activity of D14 was found to be 240% higher than that of the wild type. This could account for its high lysine producing capability, as during in vitro inhibition studies carried out on the aspartate kinase of D14 it was found not to be resistant to repression by L-lysine plus L-threonine.

Thus the AEC^R mutation of D14 enabling it to produce high lysine yields appears to be at the level of enzyme synthesis rather than the ability of the enzyme aspartate kinase to overcome repression by lysine plus threonine in the lysine biosynthetic pathway.

The regulation of aspartate kinase and the possibility of the existence of aspartate kinase isoenzymes has been investigated in a number of strains. Stadtman et al, 1954 (Tosaka and Takinami 1978) discovered that E.coli had two different and separable aspartate kinases. One is specifically and competitively inhibited by lysine and the other is specifically and competitively inhibited by threonine. There is also

evidence for the existence of a third aspartate kinase which is specifically repressed by methionine.

Shiio and Miyajima (1969) and Nakayama et al (1966) working with B.flavum and C.glutamicum respectively, reported on aspartate kinases which were inhibited only when L-lysine and L-threonine were added simultaneously. Tosaka and Takinami (1978) found that in B.lactofermentum the aspartate kinase was inhibited 45% by either L-lysine or L-threonine and 80% by L-lysine plus L-threonine. Tosaka et al (1978) obtained AEC^R mutants from B.lactofermentum and found that the aspartate kinase of these mutants was not inhibited by L-threonine alone but was inhibited by L-lysine alone. However, no concerted inhibition was obtained with L-lysine plus L-threonine. They suggested that a lysine sensitive aspartate kinase exists in aspartate kinase isoenzymes.

In the present study of the aspartate kinase of B.lactofermentum ATCC 13869, approximately 80% inhibition by L-lysine plus L-threonine and 40% inhibition by L-lysine alone was found. However no inhibition was caused by L-threonine alone and in fact stimulation occurred. It could be hypothesized that at least three isoenzymes of B.lactofermentum aspartate kinase exist. One specifically inhibited by L-lysine plus L-threonine, one by L-lysine and one by L-threonine. The relative levels of these aspartate kinase isoenzymes could change either during the growth cycle or they could possibly be repressed or stimulated according to the media the bacteria are grown in. Rosner and Paulus, (1971) found when working with Bacillus subtilis that two separate aspartate kinases could be isolated, one of which is specifically inhibited by meso-diaminopimelate (AKI) and the other (AKII), which is subject to inhibition by L-threonine plus L-lysine. In stationary cultures and in rich media, levels of AKII were found to be repressed 5 to 10 fold.

This could explain the apparent discrepency between the present results and those obtained by Tosaka and Takinami (1978) as they

always grew B.lactofermentum in a minimal medium prior to obtaining crude enzyme extracts for studies. However for all experiments carried out in this study concerning enzyme activities, B.lactofermentum ATCC 13869 was grown in a relatively rich medium. These conditions could possibly repress the threonine sensitive aspartate kinase isoenzyme. However more work is necessary to test this hypothesis fully.

During optimization studies on D14, yeast extract proved to be a particularly good source of amino acids and the value of incremental feeding was demonstrated. In a 20 ℓ laboratory fermenter trial, D14 produced 32 mg/mℓ L-lysine.HCI within 73 hours. This is a most encouraging result and although outside the scope of the present study good possibilities for further improvement through optimization exist for this mutant. The process must then be transferred to a pilot scale fermenter and the use of cheaper production media investigated. For example, corn steep liquor could be used in place of yeast extract and starch hydrolysate or molasses used as a replacement for glucose. In the case of molasses problems may arise as this product, although one of the cheapest carbon sources for fermentation processes, often contains materials toxic to bacterial growth, including the coryneform group. (Misra et al, (1980). Furthermore lysine producing mutants are highly individualistic in their response to lysine production on molasses in comparison to glucose.

In the future as the price of molasses continues to excalate, other carbon sources such as ethanol or acetic acid (Pelechová et al, 1980) may have to be considered.

If the current lysine yields obtained in this study can be maintained using production media on a large scale eg. in production fermenters of up to 200 000 litres volume (Eveleigh, 1981), a crude grade spray dried lysine powder of 25% lysine content could be produced economically as a high lysine animal feed supplement. (Wang, 1979). In this way direct competition with the purified product presently imported from Japan could be avoided.

Process optimization is one method of further increasing lysine yields in this highly competitive industrial market. Another is to continue searching for novel mutations which would facilitate high lysine yields of selected microorganisms. The progress in molecular biology especially in the understanding of the regulatory mechanics in amino acid biosynthesis by bacteria served as the initial stimulus in developing this field. The genetic techniques most often used are limited to conventional mutational methods and selection programmes. They still include the isolation of analogue resistant mutants and the selection for specific auxotrophic mutants.

However, one novel aspect was the introduction of flouropyruvate-sensitive mutants (Tosaka et al, 1981 and Ozaki et al, 1982). These mutants are sensitive to the build up of pyruvate in their biochemical pathway and phosphoenol pyruvate is rapidly channelled into lysine via the aspartate pathway. These mutants show no measurable homoserine dehydrogenase activity as is also the case with homoserine auxotrophic mutants. Thus flouropyruvate sensitivity is analogous to the homoserine auxotrophic genotype and is usually coupled to analogue resistant strains as high concentrations of lysine would inhibit the aspartate pathway thereby causing a build up of pyruvate.

Flouropyruvate sensitivity would therefore probably not contribute significantly towards increasing the lysine production potential of D14. However, this genotype could further increase the lysine production of a good AEC resistant mutant.

Other genetic techniques such as transduction, transformation, conjugation and plasmid transfer have seldom been used because such genetic systems were not generally available for most industrially important bacteria.

However, Momose et al (1976) while working with strains of coryneform and unidentified glutamic acid producing bacteria isolated twenty-four temperate phages, two of which could serve as transducing phages. This work was done in order to develop a transduction system for Brevibacterium species.

Work on plasmid transfer was carried out by Kaneko and Tanaka (1979) who isolated and characterized a plasmid from B.lactofermentum. They postulated that this plasmid could be a candidate for the construction of a mini plasmid through cleavage by various restriction enzymes and ligation.

Protoplast fusion as a genetic technique was investigated by Kaneko and Sakaguchi (1979), Peberdy (1980), Santamaria et al (1982), Tosaka et al (1982) and Zhdanova et al (1982). Protoplasts were obtained from coryneform bacteria with a view to genetic recombination by protoplast fusion. In many cases however, these recombinants showed an elevated genetic unstability.

Lereverend et al (1982) attempted to further improve a lysine-overproducing E.coli mutant using recombinant DNA techniques. The E.coli mutant they utilized had been improved by increasing separately the copy number of every gene of the lysine biosynthetic pathway (after cloning on a plasmid). This strain was further improved by cloning a gene coding for a desensitized and constitutively expressed aspartate kinase III, the first step of this pathway. Further cloning involved a Lys A gene in which a mutation allows constitutive expression of DAP-decarboxylase the last step of the pathway. In such a strain a mutation was also introduced which affects lysine - decarboxylase activity, thus decreasing lysine catabolism. Similar experiments could possibly be performed with Corynebacterium and other related genera.

These attempts at applying the relatively new developments in microbial genetics including protoplast fusion, gene amplification and recombinant DNA technology to improve lysine yielding microorganisms could possibly result in a new range of high lysine producing mutants.

However, to date, conventional mutational selection techniques have proved to be more than adequate in obtaining a wide range of high lysine producing microorganisms.

APPENDIX A

Patent List

Ajinomoto inc. (1970)
Method of producing L-lysine
British Patent 1 200 353.

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Verfahren zur Herstellung von Futterkonzentrat des L-lysins
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German Offen Patent 2 730 964.

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Mikrobiologisches Verfahren zur Herstellung flussigen und trockenen Futterkonzentrates von L-lysin und kristallinen L-lysins
German Offen Patent 2 401 519.

Viesture, Z.A., T.M. Salmone and U.E. Viestur (1974)
Microbiological method of producing a liquid or dry feed concentrate of L-lysine or crystalline L-lysine
British Patent 1 439 121.

Worgan, J.T. (1967)
Production of fermentation and cultivation media
British Patent 1 220 807.

Yukawa, H., K. Osumi, T. Nara, and Y. Takayama (1981)
Production of L-amino acids
U.S. Patent 4 276 380.

APPENDIX B

Sterilization of all media was by autoclaving for 15 min at a pressure of 15 psi unless stated otherwise.

C.glutamicum ATCC 21526 (U.S. Pat. 3 708 395)

Seed medium

Glucose	40,0 g
Polypeptone	20,0 g
KH_2PO_4	1,5 g
K_2HPO_4	0,5 g
$MgSO_4.7H_2O$	0,5 g
Biotin	50,0 µg
Urea	3,0 g
Yeast Extract	5,0 g
H_2O (distilled)	1,0 ℓ

pH 7,2

Fermentation medium

Blackstrap molasses (calculated as glucose)	100 g
Soybean Meal Hydrodyzate	20,0 g
KH_2PO_4	0,7 g
$MgSO_4.7H_2O$	0,5 g
Urea	3,0 g
$(NH_4)_2SO_4$	5,0 g
$CaCo_3$	30,0 g
H_2O (distilled)	1,0 ℓ

pH 7,5

Soybean Meal Hydrolyzate (Gutcho, S.J. 1973)

Soybean meal	250 g
Sulphuric acid (conc.)	89,67 mℓ
H_2O (distilled)	885,0 mℓ

Mix and autoclave at 120 °C (15 psi) for 4 h.
Cool, adjust pH to 7,0 with 28% NH_4OH.
Adjust final volume to 2,0 ℓ.

C.glutamicum ATCC 21513 (U.S. Pat. 3 959 075)

Seed medium

Glucose	40,0 g
KH_2PO_4	0,5 g
K_2HPO_4	1,5 g
Urea	3,0 g
$MgSO_4.7H_2O$	0,5 g
Peptone	20,0 g
Meat extract	5,0 g
Biotin	50,0 µg
H_2O (distilled)	1,0 ℓ

pH 7,2 before steralization

Fermentation medium

Blackstrap molasses (calculated as glucose)	150,0 g
$MgSO_4.7H_2O$	0,3 g
KH_2PO_4	0,7 g
Urea	3,0 g
*Ajieki (Ajinomoto inc., Japan)	20,0 g
H_2O (distilled)	1,0 ℓ

(pH 7,4 before steralization)

*Trade name for soybean acid hydrolyzate.
Previous recipe for soybean meal hydrolyzate utilized.

B. lactofermentum ATCC 21086 (U.S. Pat. 3 527 672)

Fermentation medium

Glucose	130,0 g
$(NH_4)_2SO_4$	50,0 g
KH_2PO_4	1,0 g
$MgSO_4.7H_2O$	0,4 g
Fe^{2+}	2 ppm
Mn^{2+}	2 ppm
*Amino acid powder	120 mg
DL-Threonine	500 mg
DL-Methionine	3,0 g
L-isoleucine	100 mg
L-valine	300 mg
Biotin	300 µg
Thiamine.HCl	500 µg
$CaCO_3$	50,0 g
H_2O (distilled)	1,0 ℓ

*Bacto-vitamin free casamino acids were used in place of "Amino acid powder". The total N_2 content was 0,07 g/g as was specified for the "amino acid powder".

The medium was sterilized at 120 °C (15 psi) for 10 min.

Basal medium for determining glutamic acid production

Glucose	36,0 g
Urea	10,0 g
KH_2PO_4	1,0 g
$MgSO_4.7H_2O$	0,4 g
$FeSO_4.7H_2O$	10,0 mg
$MnSO_4.4H_2O$	8,13 mg
Thiamine HCl	100 µg
*Meiki (Ajinomoto, Japan)	1,0 mℓ
H_2O (distilled)	1,0 ℓ
pH 7,0	

*Trade name for soybean acid hydrolyzate; previous recipe for soybean meal hydrolyzate utilized.

Biotin was added as required from stock solutions. This medium was filter sterilized.

Nutrient Broth

Nutrient broth (Biolab)	16,0 g
H_2O (distilled)	1,0 ℓ

0,05 M phosphate buffer pH 8,0

0,05 M NaH_2PO_4	26,5 mℓ
0,05 M Na_2HPO_4	473,5 mℓ

Dilute to 1,0 ℓ with H_2O (distilled)

Nutrient Agar

Nutrient Agar (Biolab)	31,0 g
H_2O (distilled)	1,0 ℓ

Minimal medium (medium 2)

Glucose	20,0 g
$(NH_4)_2SO_4$	10,0 g
Urea	2,5 g
KH_2PO_4	1,0 g
$MgSO_4.7H_2O$	0,4 g
$FeSO_4.7H_2O$	2,0 g
$MnSO_4.4 - 6H_2O$	2,0 g
Biotin	50 µg
Thiamine.HCl	100 µg
*Agar	10,0 g
H_2O (distilled)	1,0 ℓ

*When required

Amino acid supplements to medium 2

L-Homoserine	600 mg/ℓ
L-leucine	20 mg/ℓ

Additional supplements when screening for analogue resistance

L-Threonine	476 mg/ℓ
AEC	4 mM
L-lysine hydroxamate	4 mM
N- -methyl-L-lysine	4 mM

(These were all filter sterilized.)

Screening medium for testing L-lysine producing ability of Corynebacterium and Brevibacterium

Seed medium

Glucose	20,0 g
Peptone	10,0 g
Meat extract	5,0 g
NaCI	3,0 g
Biotin	10 µg
H_2O (distilled)	1,0 ℓ

Fermentation medium

Glucose	75,0 g
$(NH_4)_2SO_4$	15,0 g
K_2HPO_4	0,5 g
KH_2PO_4	0,5 g
$MgSO_4.7H_2O$	0,25 g
Biotin	30 µg
*NZ-amine	5,0 g
$CaCO_3$	10,0 g
H_2O (distilled)	1,0 ℓ

*NZ-amine = Bactocasitone = casein enzyme hydrolyzate.

Medium I

Polypeptone	10,0 g
Yeast extract	10,0 g
NaCl	5,0 g
**Agar	20,0 g
H_2O (distilled)	1,0 ℓ

Adjust to pH 7,0

** When required.

0,1 M sodium phosphate buffer pH 7,0

0,05 M Na_2HPO_4	- A
0,05 M $NaH_2PO_4.H_2O$	- B

Add solution B to 500 mℓ of solution A until pH 7,0 is reached.

TNI Screening medium

Seed medium

As in testing L-lysine producing ability of Corynebacterium and Brevibacterium.

Fermentation medium (TNI)

Glucose monohydrate	110 g
$(NH_4)_2SO_4$	40,0 g
KH_2PO_4	1,0 g
$MgSO_4.7H_2O$	0,4 g
$FeSO_4.7H_2O$	10,0 mg
$MnSO_4.4H_2O$	8,13 mg
Thiamine.HCl	200 µg
NZ-amine	10,0 g

Biotin	50,0 µg
$CaCO_3$	50,0 g
H_2O (distilled)	1,0 ℓ

Adjust to pH 7,2 with 1 M NaOH

Mixture for eluting L-lysine spots from the paper chromatogram

Methanol	75,0 mℓ
H_2O (distilled)	24,0 mℓ
0,5% ($^w/_v$) aqueous solution of $CuSO_4.5H_2O$	1,0 mℓ

TNIY screening medium

Seed medium

Glucose	20,0 g
Peptone	10,0 g
Meat extract	5,0 g
NaCl	3,0 g
Biotin	10 µg

Fermentation medium (TNIY)

Glucose monohydrate	110,0 g
$(NH_4)_2SO_4$	40,0 g
KH_2PO_4	1,0 g
$MgSO_4.7H_2O$	0,4 g
$FeSO_4.7H_2O$	10 mg
$MnSO_4.4H_2O$	8,13 mg
Thiamine.HCl	200 µg
Biotin	50 µg
Yeast extract	10,0 g
$CaCO_3$	50,0 g
H_2O (distilled)	1,0 ℓ

pH adjusted to 7,2 with 1 M NaOH

APPENDIX C

Sample: Polypeptone (BBL)

Amino Acid	mg/g sample
Alanine	6,9
Arginine	18,9
Asparagine	3,8
Glutamine	7,8
Glycine	4,2
Isoleucine	7,8
Leucine	37,0
Lysine	29,0
Methionine	7,9
Phenylalanine	17,5
Proline	Trace
Serine	4,9
Threonine	4,9
Tryptophan	ND*
Tyrosine	6,0
Valine	8,5
Total	165

* ND = Not detected.

Sample : Yeast Extract (Merck)

Amino Acid	mg/g sample
Alanine	41,8
Arginine	10,7
Asparagine	34,5
Cysteine	6,6
Glutamine	47,8
Glycine	18,0
Histidine	15,9
Isoleucine	25,0
Leucine	54,2
Lysine	40,3
Methionine	13,5
Phenylalanine	29,5
Proline	10,8
Serine	23,2
Threonine	22,3
Tryptophan	ND*
Tyrosine	16,6
Valine	32,5
Total	443,2

* ND = not detected.

Sample : Proteose Peptone (Difco)

Amino Acid	mg/g sample
Alanine	5,3
Arginine	1,2
Asparagine	1,9
Glutamine	5,2
Glycine	5,2
Histidine	Trace
Isoleucine	2,7
Leucine	9,8
Lysine	8,7
Methionine	3,5
Phenylalanine	2,5
Serine	3,1
Threonine	2,5
Tryptophan	ND*
Tyrosine	2,0
Valine	Trace
Total	53,6

* ND = not detected.

Sample : Corn Steep Liquor

Amino Acid	mg/mℓ sample
Alanine	4,5
Arginine	2,9
Asparagine	3,1
Glutamic acid	0,6
Glutamine	4,2
Glycine	1,5
Histidine	0,8
Isoleucine	1,5
Leucine	7,4
Lysine	2,3
Methionine	1,8
Phenylalanine	4,6
Proline	5,4
Serine	2,4
Threonine	2,0
Tryptophan	ND*
Tyrosine	4,8
Valine	1,8
Total	52,9

* ND = not detected.

Sample : Soyabean Hydrolysate

Amino Acid	mg/mℓ sample
Alanine	1,0
Arginine	1,2
Asparagine	3,4
Cysteine	0,3
Glutamine	3,6
Glycine	1,3
Histidine	0,3
Isoleucine	0,3
Leucine	1,0
Lysine	1,0
Phenylalanine	0,6
Proline	1,0
Threonine	0,5
Serine	1,0
Tyrosine	0,4
Valine	0,1
Methionine	0,3
Total	17,3

Sample : Bacto-Casitone (Difco)

Amino Acid	mg/g sample
Alanine	8,4
Asparagine	4,3
Glutamine	12,4
Glycine	2,1
Histidine	4,7
Isoleucine	9,5
Leucine	49,0
Lysine	27,9
Methionine	11,4
Phenylalanine	26,7
Serine	7,8
Threonine	7,5
Tyrosine	6,6
Valine	10,9
Total	189,2

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